STUDIES ON SOME UNUSUAL CHARACTERISTICS EXPRESSED BY PSEUDOMONAS AERUGINOSA ASSOCIATED WITH CHRONIC RESPIRATORY INFECTIONS

by

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ABSTRACT

Patients with compromised respiratory host defences, particularly associated with cystic fibrosis (CF), are susceptible to respiratory tract colonization by Pseudomonas aeruginosa. Isolates of P. aeruginosa from the sputum of patients suffering from chronic, obstructive pulmonary disease express one or more unusual characteristics. These include a mucoid colonial morphology (due to alginate synthesis), antibiotic hypersensitivity, loss of the specific O-antigen, polyagglutinability and serum sensitivity.

Problems associated with the epidemiological typing of these atypical strains were overcome by the development of a technically improved spotting method of pyocin typing, based on the method of Gillies & Govan (1966). The spotting method provides greater discrimination through the recognition of S-pyocin production, and confirmed that in most cases, the phenotypic heterogeneity of P. aeruginosa isolates from CF patients emerged in vivo.

The genetic basis of alginate synthesis and antibiotic hypersensitivity in P. aeruginosa were investigated using two different approaches. In the case of alginate synthesis, mucoid derivatives of P. aeruginosa strain PAO were isolated and at least two loci (muc) associated with the "switching on" of alginate synthesis were mapped on the chromosome by means of FP2-mediated conjugation and F116L-mediated transduction. Evidence for the regulatory nature of one of these muc loci was obtained following the construction of R'muc and R'muc$^+$ plasmids from R68.45. The R'muc$^+$ plasmid, pJF4, "switched off" alginate synthesis in four out

of five clinical mucoid P. aeruginosa isolates, suggesting that the muc locus characterized in PAO is responsible for "switching on" alginate synthesis in some clinical strains.

The genetic basis of clinically associated antibiotic hypersensitivity was investigated using P. aeruginosa isolates 492a and 492c. These strains were from the same sputum specimen and of the same pyocin type, but expressed different antibiograms. 492c was hypersensitive (10-100 times more sensitive than 492a) to carbenicillin, methicillin, flucloxacillin, mecillinam, cefuroxime and naladixic acid, and showed enhanced sensitivity (4-8 times more sensitive than 492a) to chloramphenicol, trimethoprim and novobiocin. Two genetic determinants associated with antibiotic hypersensitivity (blsA1 and tpsA1) were mapped in the 30 min region of the chromosome by means of R68.45-mediated plate matings between a Leu$^-$ mutant of 492c and PAO recipients, followed by transductional analysis using F116L. blsA1 was closely linked to nalB and was responsible for the difference in antibiograms between 492a and 492c, whereas tpsA1, which mapped between ser-3 and hisV could also be transferred to PAO from 492a. The genetic basis of antibiotic hypersensitivity in the unrelated clinical isolates 519c and 2358 was similar to that demonstrated for 492c. A blsA mutant was isolated from PAO969 following mutagenesis and screening.

Using a similar approach, two genetic determinants associated with antibiotic hypersensitivity (blsB3 and blsC4) were identified in the mutant strain 799/61. blsB3 was cotransducible with pro-82 and coded for hypersensitivity to β-lactams, trimethoprim, naladixic

acid, novobiocin, rifampicin and chloramphenicol. blsC4 was closely linked, but distinct from blsA and gave rise to a similar phenotype in PAO as that associated with blsA.

CONTENTS

DECLARATION

The experiments and composition of this thesis are entirely the work of the author

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INTRODUCTION

CHAPTER I

PSEUDOMONAS AERUGINOSA: THE ORGANISM AND ITS HABITAT

Members of the genus Pseudomonas are widespread microbial inhabitants of soil and water (Douderoff & Palleroni, 1974). They are Gram negative straight or curved rods with dimensions 0.5 to 1μm by 1.5 to 4μm, and are motile by means of single or multiple polar flagella (Palleroni, 1975). They are strict aerobes, except for those species which can use denitrification as a means of anaerobic respiration, and are amongst the most catabolically versatile microbes known.

The genus can be subdivided into five groups on the basis of rRNA homology (Palleroni, 1975). rRNA Homology Group I contains the fluorescent species of which Pseudomonas aeruginosa is the type species. Strains of P. aeruginosa are generally pigmented due to the production of pyocyanin and various fluorescent pigments, and grow well at 37°C (a characteristic which distinguishes them from other fluorescent pseudomonads) with an upper limit in growth temperature of 44°C (Palleroni, 1975). P. aeruginosa is a nutritionally versatile species and most strains are capable of utilizing 70 to 80 organic compounds as growth substances (Douderoff & Palleroni, 1974). According to Rhame (1980) "P. aeruginosa is a strong contender for the microorganism capable of thriving in the greatest number of seemingly dissimilar ecological niches."

P. aeruginosa is generally a saprophyte and appears to be a natural inhabitant of agricultural soil. Green et al (1974) recovered P. aeruginosa from 34% of 58 soil samples taken from different Californian locations where there was no history of the use of organic fertilizers, no pasturing of animals and no P. aeruginosa isolated from the irrigation water. Soil in which tomatoes were grown yielded the most frequent isolations (46% of 24 samples). In another study, Schroth et al (1977) demonstrated that P. aeruginosa could frequently be isolated from soil used in pots for ornamental plants (84% of 49 soil samples).

The plants themselves are only rarely colonized in the field (Green et al, 1974), although under conditions of moderately high temperatures and humidity, P. aeruginosa will survive and multiply within plant tissue, causing rotting of the leaves. This is a particular problem in greenhouses (Schroth et al, 1977), and in the tropics where P. aeruginosa is the cause of an economically important disease of tobacco (Elrod & Braun, 1942). Once harvested, salad plants in particular are rapidly colonized with P. aeruginosa (Shooter et al, 1969; Kominos et al, 1972), probably due to the humid conditions usually inherent in transport and storage.

Perhaps the most important habitat for P. aeruginosa is surface water, particularly where there has been human or animal activity. This is partly due to the fact that a small proportion of normal human beings (8% according to Stoodley & Thoma, 1970) and domestic animals (3% according to Ringen & Drake, 1952) carry P. aeruginosa as part of their normal gut flora. Consequently, P. aeruginosa is frequently isolated from sewage and polluted streams (Hoadley,

1977). In warm climates, the species also thrives in organically rich unpolluted stream water (Hoadley, 1977). Swimming pool water is frequently contaminated with P. aeruginosa, particularly in the absence of adequate chlorination (Némedi & Lányi, 1971), likewise jacuzzis or health spas (Vogt et al, 1982).

As if to emphasize its aquatic predilection and versatility, P. aeruginosa can frequently be isolated from distilled water where populations of up to 10^7 cells ml^{-1} can be maintained for up to 42 days (Favero et al, 1971).

Although P. aeruginosa can be isolated from seawater, particularly near sewage outfalls, it is probably not naturally a marine organism (Hoadley, 1977).

As well as its nutritional versatility, P. aeruginosa is reknowned for its intrinsic resistance to a wide range of antimicrobial agents and this characteristic extends its natural habitat considerably. The organism is able to survive and often multiply in aqueous quaternary ammonium disinfectants (Neu, 1983) as well as soaps, handcreams, ophthalmological solutions and infusion fluids. Consequently, the hospital environment provides an ideal habitat for P. aeruginosa due to the constant provision of moisture, warmth and a source of nutrients generally unsuitable for most other organisms.

CHAPTER II

HOST FACTORS ASSOCIATED WITH *P. AERUGINOSA* COLONIZATION AND INFECTION

As previously mentioned, up to 8% of the normal human population may carry *P. aeruginosa* as a gut commensal, but, in general, healthy individuals are not colonized by this organism either in the gastrointestinal tract or any other site. However, hospitalized patients, particularly those being treated in an intensive care unit, rapidly become colonized by *P. aeruginosa*, and the frequency of intestinal carriage in such patients can be as high as 75% (Neu, 1983). Likewise, oropharyngeal colonization by *P. aeruginosa* and other Gram negatives, normally a rare occurence (Rosenthal & Tager, 1975), seems to increase with severity of illness (Johanson *et al*, 1972).

To a normal, healthy individual *P. aeruginosa* is not a particularly invasive organism, but to a compromised individual, the species is an opportunitist pathogen *par excellence*, and like a microbial hyena will lurk in the background waiting to attack those less able to defend themselves.

No single factor is of paramount importance in host defence against *P. aeruginosa*, as an effective system is dependent on the integrated functions of both local and systemic components (Peterson, 1980). Breakdown of the local defences at any epithelial surface can lead to colonization by *P. aeruginosa*, and the consequences of

such colonization are dependent on the state of the remaining, systemic defence mechanisms, and the virulence of the particular bacterial strain.

The increased susceptibility of hospitalized patients to P. aeruginosa colonization and infection follows the breakdown of various components of the normal host defences through injury, prior illness, therapy or surgical procedures. However, healthy individuals can increase their susceptibility to P. aeruginosa colonization and infection through a variety of occupational or recreational pursuits, e.g. deep-sea diving under saturation conditions (Alcock, 1977) and prolonged submersion in a jacuzzi (Sausker et al, 1978; Vogt et al, 1982).

The range of infections attributed to P. aeruginosa is extensive and has been reviewed elsewhere (Cross et al, 1983; Geddes, 1980; Neu, 1983; Sherertz & Sarubbi, 1983). The purpose of this chapter is to consider the factors associated with P. aeruginosa colonization and infection in one particular site - the respiratory tract.

P. aeruginosa is associated with two major forms of respiratory tract infection. The first of these is acute pneumonia, which is the most common of the nosocomial infections caused by this organism (Sherertz & Sarubbi, 1983), and usually affects the elderly (Rose et al, 1973), the immunocompromised patient (Reynolds & Fick, 1980), or the patient requiring prolonged treatment in an intensive care unit (Johanson et al, 1972). In some instances, pneumonia due to P. aeruginosa can be acquired outside hospital, particularly by

individuals with chronic lung or heart disease (Tillotson & Lerner, 1968). Many cases of P. aeruginosa pneumonia develop as a further complication of bacteraemia, particularly in leukaemic patients (Neu, 1983).

The second type of respiratory tract infection involving P. aeruginosa is a chronic pulmonary infection usually associated with cystic fibrosis (CF) patients (Doggett & Harrison, 1969), and occasionally causing exacerbations in chronic bronchitics (Rivera & Nicotra, 1982). Unlike acute pneumonia which is often associated with bacterial invasion and dissemination, pulmonary infection in CF patients is a localized bronchiolitis and the mechanism of tissue damage appears to be different (Høiby, 1984).

Prior colonization is an important predisposing factor in the acquisition of both these kinds of respiratory infection. In the case of nosocomial pneumonia, Johanson et al (1972) observed that 23% of patients colonized with Gram negative bacteria, later developed pneumonia, compared with 3% of the non-colonized patients. Prior colonization with the causative organism was demonstrated in 91% of patients who developed nosocomial pneumonia. The percentage of CF patients colonized with P. aeruginosa varies from one centre to another, and estimates range from 50% (Høiby, 1982; Kulczycki et al, 1978) to 90% (Elston & Hoffman, 1967). The transition from asymptomatic P. aeruginosa colonization to chronic and progressive pulmonary infection in these patients is difficult to define and may vary from one patient to another, however, it is likely that both host and bacterial factors are involved (Høiby, 1974).

The remainder of this chapter will discuss the host factors associated with P. aeruginosa colonization and infection in the respiratory tract. The bacterial factors associated with the pathogenesis of P. aeruginosa pneumonia and chronic pulmonary infection will be the subject of the next chapter.

P. aeruginosa colonization of the respiratory tract

Figure 1 illustrates the human respiratory tract indicating the anatomy and major components of normal respiratory defence mechanisms. These mechanisms can be divided into four categories (Reynolds, 1983).

(1) Mechanical; e.g. the cough reflex, which in normal subjects is a very effective means of expelling foreign bodies and secretions (Newhouse et al, 1976); and the mucociliary clearance mechanism which is important between the posterior two-thirds of the nasal cavity and nasopharynx, and the larynx to the terminal bronchioles (Newhouse et al, 1976).

(2) Anatomical, e.g. the nose, epiglottis, larynx and respiratory branch which are responsible for directing the air-flow.

(3) Non-specific antimicrobial and antitoxic factors present in tracheobronchial secretions and on the alveolar surfaces, e.g. α_1-antitrypsin, which is inhibitory to bacterial proteases, iron-binding proteins (lactoferrin and transferrin), and surfactants (Reynolds & Fick, 1980).

(4) Immunological mechanisms, e.g. immunoglobulins, complement components and alveolar macrophages (Reynolds, 1983).

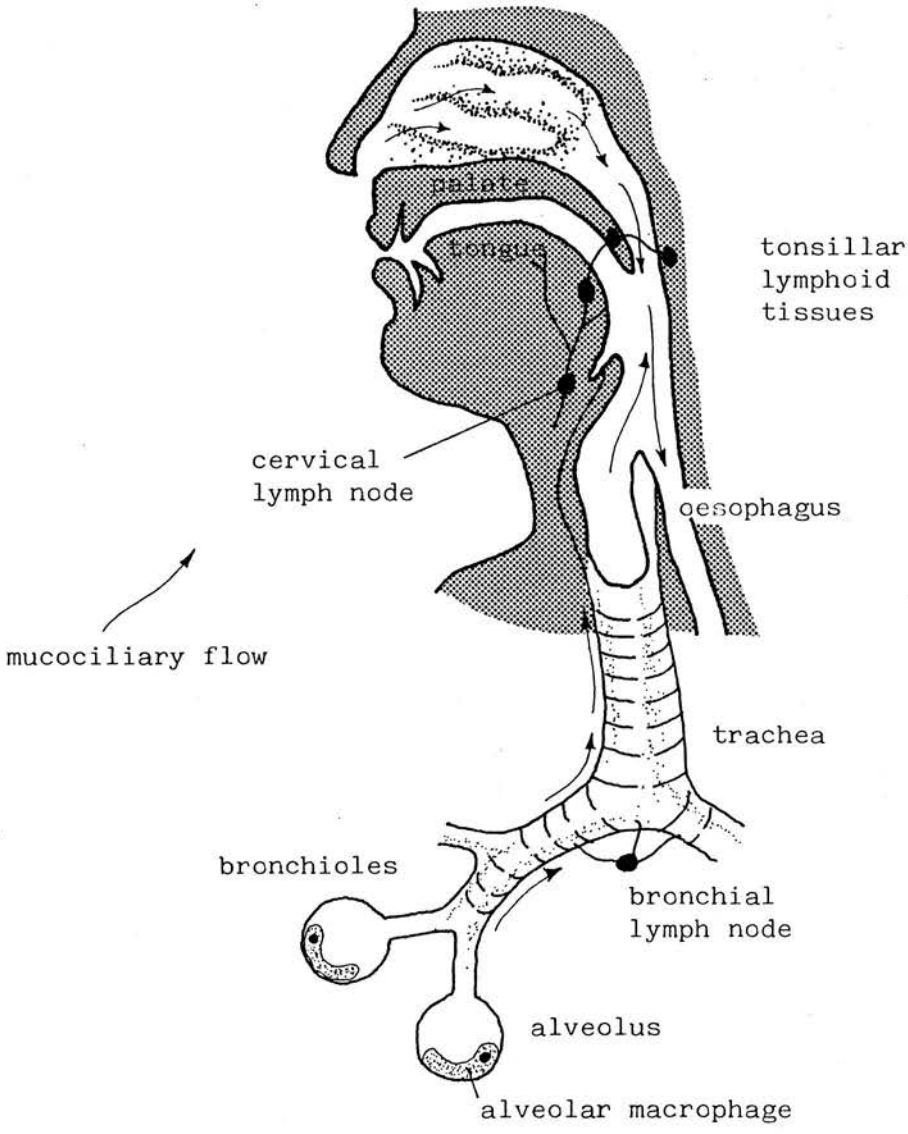


Figure 1. The normal host defences of the respiratory tract (Based on Mims, 1982).

These mechanisms combine to provide surveillance of the respiratory tract under normal circumstances. However, in times of crisis, the responsiveness of the system can be enhanced by the recruitment of various components of the systemic defences, i.e. mobilization of polymorphonuclear granulocytes (PMN) to the lung tissue, providing a secondary supply of phagocytes. In addition, alteration of the permeability of the alveolar surfaces as a result of inflammation allows the entry of intravascular proteins, further complement components, antibodies and other immune constituents (Reynolds & Fick, 1980).

Only 2% of normal individuals carry P. aeruginosa as part of their normal pharyngeal flora (Rosenthal & Tager, 1975), however, the upper respiratory tract is extremely susceptible to P. aeruginosa colonization in severely ill patients (Johanson et al, 1972). The reasons for this colonization are not entirely clear, but the nutritional status of the patient is probably important (Reynolds, 1983). It has been suggested that the normal resistance of upper respiratory tract epithelial cells to Gram negative colonization is associated with the presence of a particular cell-surface glycoprotein, fibronectin (Woods et al, 1983). Fibronectin is thought to mask Gram negative bacterial receptor sites on the epithelial cell surface, and factors associated with severe or chronic illness reduce the level of fibronectin, thus increasing the availability of these receptor sites, and facilitating colonization by P. aeruginosa or other Gram negatives (Johanson et al, 1979). Fibronectin also acts as a binding site for Gram positive organisms

whose presence normally prevent colonization by Gram negatives (Sprunt & Redman, 1968).

It is apparent that conditions impairing lung clearance promote bacterial colonization (Johanson et al, 1972). Clearance of particulate material from the airways is the raison d'etre of the mucociliary escalator, the normal functioning of which requires normal beating cilia, a periciliary fluid layer in which the cilia beat and the presence of a material with special rheological properties to act as a mechanical coupler, i.e. airway mucus (Lopez-Vidriero, 1981). The mucus is a complex secretion from secretory cells of the submucosal layer and surface epithelium, substances locally produced and tissue fluid transudate (Lopez-Vidriero, 1981). It is 95% water with the glycoprotein, mucin, as the main macromolecular component. Other components include lipids, immunoglobulins, lysozyme, lactoferrin, proteolytic enzymes and protease inhibitors. The rheological properties of the mucus are determined by the special characteristics of the mucin molecule and the degree of cross-linking between mucin molecules, the protein components also contribute (Lopez-Vidriero, 1981). Mucus is released on to the surface of the ciliated epithelium as droplets and these are drawn out into strands by the action of the cilia which beat in the periciliary zone of fluid of low viscosity (Sleigh, 1981). If this periciliary layer is too shallow or too deep, the propulsion of mucus will be severely restricted or may cease altogether (Sleigh, 1981). In general, both the rate of ciliary beat and proportion of ciliated cells in the epithelium increase from smaller to larger

airways so that the role and efficiency of mucus propulsion increases as it ascends the mucociliary escalator (Sleigh, 1981).

Individuals suffering from the "immotile cilia syndrome", a congenital defect in the structure and function of respiratory and other cilia, are prone to chronic respiratory infections (Eliasson et al, 1977) presumably following bacterial colonization.

It has recently been shown, using an in vitro system, that P. aeruginosa binds extremely well to human tracheobronchial mucin (Vishwanath & Ramphal, 1984), suggesting that airway mucus may be an important colonization site for P. aeruginosa in individuals with defective mucociliary clearance, e.g. patients with CF, who will be discussed in the following section.

The role of the immunological components of the respiratory host defences in prevention of bacterial colonization has not been well documented. However, in one study, Brownstein (1978) showed the immunosuppression of mice rapidly led to upper respiratory tract colonization by P. aeruginosa followed by pneumonia.

Tracheostomy or introduction of an endotracheal tube can lead to bacterial colonization of the tracheobronchial tree in the same way that catheterization can lead to colonization of the urinary tract. Particularly if ventilatory equipment is attached which may itself be contaminated, and as a result, forcefully aerosolize the bacteria into the airways (Reynolds & Fick, 1980). This factor is particularly relevant in the case of P. aeruginosa, considering the organism's ability to colonize hospital equipment and to contaminate instruments and prostheses. Alternatively, the bacterial source may

be the patient's own pharyngeal or gastric secretions (Sanderson, 1983).

P. aeruginosa colonization of the respiratory tract in CF patients

Cystic fibrosis is the most frequent lethal genetic syndrome among caucasian children (Wood et al, 1976), and is inherited as an autosomal recessive trait (Danks et al, 1965). The incidence of CF varies between populations, ranging from 1 in 620 live births in South West African Afrikaners of Dutch descent (Super, 1975) to 1 in 15,000 amongst the Italians (Antonelli & Donfrancesco, 1970). In middle and western Europe, the incidence is approximately 1 in 2,000 (Wood et al, 1976). CF has been detected in non-white populations, though the incidence is very low, e.g. amongst blacks in Washington D.C., the frequency is 1 in 17,000 live births (Kulczychi & Schauf, 1974).

No known biochemical or structural defect accounts for all the pathophysiologic phenomena associated with CF, thus its definition rests on the clinical findings. Generally, there are four criteria for diagnosis: (i) A positive sweat test (sweat chloride $>60mEql^{-1}$), (ii) Chronic obstructive pulmonary disease, (iii) Exocrine pancreatic insufficiency (in 80 to 90% of patients), (iv) Family history can be helpful if confirmed cases are known (Wood et al, 1976).

Obstruction of exocrine gland ducts occurs in all (or nearly all) patients due to the inspissation of secretions, and one of the earliest possible problems arising from this is a gastrointestinal

blockage known as meconium ileus, which originally lead to death in up to 25% of babies born with CF (Oppenheimer & Esterly, 1962).

However, the clinical features associated with CF are extremely variable in their expression and severity, and it has been estimated that 40-50% of sufferers remain undiagnosed (Fick, 1981). For those patients who are diagnosed, the average life expectancy has increased dramatically over the last 30 years. In 1950 patients invariably died in infancy but by 1976 the average life expectancy had increased to 19 years (Chartrand & Marks, 1983). Better management of gastrointestinal complications is largely responsible for this improvement, and consequently the fate of the majority of patients nowadays is determined by the progressive pulmonary disease which accounts for >95% of deaths other than those due to meconium ileus (Wood et al, 1976).

There is no simple explanation for the natural history and pathogenesis of the CF pulmonary disease, but it is generally agreed that bacterial infection is important and responsible for the irreversible lung damage (Reynolds & Fick, 1980). The lungs are apparently normal at birth (Wood et al, 1976), and it has been suggested that circulating pancreatic proteases initiate early inflammatory changes in the small airways, which subsequently become colonized by bacterial and/or viral pathogens (Kuzemko, 1983).

The bacterial species most frequently isolated from the respiratory tracts of CF patients are Staphylococcus aureus, Haemophilus influenzae and P. aeruginosa (Doggett & Harrison, 1969; Kulczycki et al, 1978; Mearns et al, 1972; Iacocca et al, 1963).

The relative significance of these organisms in the pathogenesis of lung disease has been the subject of considerable controversy. One theory is that S. aureus is always the initial pathogen, and colonisation by P. aeruginosa takes place only following lung damage (Burns & May, 1968; Lawson, 1969; Kilbourn, 1978). However, Mearns et al (1972) noted a significant decrease in the frequency of isolation of S. aureus from CF patients between 1950 and 1971, probably as a result of improved anti-staphylococcal therapy, while over the same period, P. aeruginosa isolations increased.

This raises the question of the role of antimicrobial therapy (particularly directed against S. aureus) in predisposing to P. aeruginosa colonization. Several studies have concluded that this is indeed a major contributing factor (Huang et al, 1961; Burns & May, 1968; Iacocca et al, 1963; Kulczycki et al, 1978). However, prior antimicrobial therapy is unlikely to provide the whole answer, as one of the earliest bacteriological studies of 14 CF patients , published before the era of routine broad spectrum antibiotic therapy (Di Sant'Agnese & Anderson, 1946) reported a single patient colonized by Bacillus pyocyaneus (P. aeruginosa). Similarly, Iacocca et al (1963) reported that prior antibiotic therapy merely increased the frequency of P. aeruginosa isolations from 18% to 43%. An alternative explanation for the increased frequency of P. aeruginosa isolations noted by Mearns and colleagues (1972), is the change in patient population as a result of increased life expectancy.

Prior lung damage may, as Lawson suggests (Lawson, 1969), be a key predisposing factor to P. aeruginosa colonization, however, in a more recent survey, Høiby (1982) noted that 50% of the CF patients attending his clinic in Denmark were colonized with P. aeruginosa, and that some, but not all of those had impaired lung function at the time when P. aeruginosa was first isolated.

The role of P. aeruginosa in the pathogenesis of lung disease in CF patients is not the subject of the present section and will be discussed later. Here, I am primarily concerned with those aspects of the CF respiratory tract which are associated with increased susceptibility to colonization by P. aeruginosa, assuming that prior antibiotic therapy and lung damage do not provide the whole answer.

In order to tackle this problem we need to ask (i) what part of the respiratory tract is initially colonized by P. aeruginosa?, and (ii) consider how the normal host defences are compromised in the CF patient.

Iacocca et al (1963) cultured specimens from the nasopharynx and throat, and compared them with sputum samples taken on the same occasion from 29 CF patients. P. aeruginosa was isolated from 62% of the sputum samples, 55% of the throat specimens, but only 31% of the nasopharyngeal specimens. Laraya-Cuasay et al (1976) noted that 86.6% of their patients carried P. aeruginosa in the throat, but isolates from the nose were extremely rare (as were skin isolates). Shapiro et al (1982) performed quantitative bacteriology on sinus aspirations from 20 CF patients and found that 13 yielded P. aeruginosa in numbers $> 10^4$ bacteria ml^{-1}. However, they found no

association between the bacteria isolated in the sinuses and those in the nasopharynx, throat or sputum. Woods et al (1980) demonstrated a correlation between in vitro adherence of P. aeruginosa to buccal cells and respiratory tract colonization with the organism. In this study, the P. aeruginosa adherence in vitro varied directly with the loss of fibronectin from the buccal cell surface, as well as increased levels of salivary proteases.

According to Kulczycki and colleagues (1978), P. aeruginosa flourishes in the bronchial secretions of CF patients, and this has been confirmed in vitro by Ohman & Chakrabarty (1982).

It is generally accepted that the tracheobronchial secretions of a CF patient differ from those of a normal individual, and from patients with other respiratory conditions. However, it is not always clear whether this is the cause or result of bacterial colonization and infection. Fick & Reynolds (1983) obtained lung lavage fluid and sputum from a group of CF patients and compared them with similar secretions from non-CF individuals (smokers and non-smokers). The CF secretions were found to contain more phagocytic cells, immunoglobulins, complement components, α_1-anti-trypsin and proteases, but less transferrin than the non-CF secretions. These differences could all be explained on the basis of inflammation. However, Frates et al (1983) cultured explants of airway tissue from CF patients and patients suffering from other chronic pulmonary disease. This method overcame the complications of infection, and they were able to show that the secretion rate of

mucus glycoproteins was 3-6 fold higher in CF patients than non-CF, and the CF glycoproteins were more highly sulphated and more acidic than those from non-CF tissue explants. According to Roussel (1984), CF glycoproteins have longer carbohydrate chains, and he has suggested that "an inborn alteration of bronchial mucin carbohydrate chains, inducing a specificity in bacterial adhesion, and an alteration of the mucociliary clearance represents a tempting hypothesis to explain the increased risk of infection".

The tracheobronchial secretions from CF patients have a lower water content than normal (Wood et al, 1976). This could be explained by the recent finding that epithelial cells in the respiratory tract and sweat glands have an abnormally low permeability to Cl^- (Quinton, 1983; Knowles et al, 1983). Consequently, in the case of respiratory epithelium, the balance of transepithelial liquid flow would be shifted away from the lumen, thus concentrating the mucus on the airway surfaces (Knowles et al, 1983).

The electrolyte content of CF tracheobronchial secretions appears to differ significantly from normal, e.g. Na^+ and Cl^- levels are lower and K^+ is raised (Matthews et al, 1963). There is some controversy concerning the levels of Ca^{2+} in CF secretions. There appears to be an alteration in the cellular handling of Ca^{2+} which may lead to enhanced accumulation of Ca^{2+} by the CF cell (Case, 1984), and certainly the Ca^{2+} concentration of CF saliva is raised (Martinez, 1982). In addition, the increased anionic character of the mucous glycoproteins may promote increased sequestration and release of Ca^{2+} (Wood et al, 1976).

On the basis of the altered physical properties of CF tracheobronchial secretions, in particular the reduced water content, it would be reasonable to presume that mucociliary transport would be impaired. However, there is some controversy relating to this point. According to Wood et al (1976), mucociliary transport is impaired 5 to 10 fold in CF patients. Wanner (1981) also reported a reduced tracheal mucus transport rate in 14 adult CF patients, but found no correlation between transport rate and clinical status. Yeates et al (1976) observed abnormal mucociliary transport in some, but not all CF patients.

There have been few reports on the structure and function of the ciliated epithelium in CF patients, although epithelial metaplasia with loss of cilia has been noted in young CF infants who died from non-respiratory complications (Bedrossian et al, 1976). On the other hand, there have been numerous reports (reviewed by Wood et al, 1976) of "ciliary dyskinesis factors" in the sera of CF patients and their parents. In the hands of certain workers, these factors disrupt the ciliary beat pattern of rabbit tracheal explants and oyster gill cilia. However, a similar effect on human ciliated epithelium has not been proven.

Regardless of the status of the mucociliary transport mechanism in CF patients, a direct consequence of excessive secretion of abnormal respiratory mucus, of which there is no doubt, is the plugging of peripheral airways - one of the earliest pulmonary lesions found almost universally among CF patients of all ages

(Bedrossian et al, 1976). These mucus plugs may well provide an ideal colonization site for P. aeruginosa.

There appears to be no particular immunological defect in CF patients which would predipose to P. aeruginosa colonization of the respiratory tract. The alveolar macrophages appear to be morphologically and functionally normal (Thomassen et al, 1980), phagocytosis by peripheral leucocytes is normal (Biggar et al, 1971), immunoglobulin levels appear to be normal (including secretory IgA) and no consistent complement abnormality has been reported (Wood et al, 1976).

However, once colonization has been established, various abnormalities may be associated with chronic pulmonary infection and these will be discussed in a later section.

Invasive P. aeruginosa respiratory tract infection

Colonization of the upper respiratory tract of a severely ill patient can give rise to large numbers of bacteria (10^7 organisms ml^{-1}) in oropharyngeal secretions, and impaired consciousness increases the chances of aspirating those secretions (Woods, 1983). Alternatively, the bacterial reservoir may be a colonized trachea, which is often the case in ventilated patients (Sanderson, 1983). In order to determine which components of the normal host defences are important in the control of P. aeruginosa invasion of the respiratory tract, it is necessary to determine which patients are

particularly susceptible to P. aeruginosa infection and consider the nature of the defect(s) in these patients.

In a study of 36 cases of P. aeruginosa pneumonia, Pennington et al (1973) concluded that cancer was the most common predisposing condition, followed by chronic lung or heart disease. These workers also observed that susceptibility to P. aeruginosa pneumonia was frequently associated with treatment directed towards the primary disease, e.g. cancer chemotherapy. Rose et al (1973) noted that elderly men recovering from major surgery were particularly at risk and many of these were receiving multiple or broad spectrum antimicrobial drugs prior to the onset of P. aeruginosa pneumonia. A significant number of these patients (42%) developed bacteraemia and this was invariably fatal. The major predisposing factor in the acquisition of non-bacteraemic P. aeruginosa pneumonia appears to be chronic lung or heart disease (Tillotson & Lerner, 1968) and the mortality rate, even in the absence of bacterial dissemination to the bloodstream, was 80% in this study. Crane & Lerner (1983) have quoted a 72% overall mortality rate of P. aeruginosa pneumonia.

Various experimental models have set out to determine the role of specific host defence components in the control of acute P. aeruginosa pneumonia. Pennington & Emrie (1978) showed that immunosuppressed guinea pigs invariably died from bilateral haemorrhagic pneumonia following intratracheal instillation of P. aeruginosa, and that mortality was correlated with a diminished PMN inflammatory response. Kazmierowski et al (1977) demonstrated that short term survival of experimental P. aeruginosa pneumonia could be enhanced in leucopoenic dogs, by treatment of the animals

with type-specific anti-pseudomonas IgG. However, granulocyte transfusion and antimicrobial chemotherapy were necessary for long term survival. Granulocyte transfusions have been shown to improve the chances of survival of severely granulocytopoenic human patients with established P. aeruginosa infection (Alavi et al, 1977).

From these, and other studies involving P. aeruginosa bacteraemia (Brownstein, 1978; Peterson, 1980), it appears that the granulocyte plays a significant role in controlling P. aeruginosa invasion and dissemination. The normal role of this cell type is the phagocytosis and intracellular killing of opsonized bacteria, where opsonization involves complement components and/or specific antibodies (Young & Armstrong, 1972). Non-opsonic phagocytosis in vitro has also been reported for certain P. aeruginosa strains (Speert et al, 1984).

Conditions leading to quantitative and or qualitative granulocyte abnormalities include acute leukaemia (Aduan & Reynolds, 1979) and burns (McEuen et al, 1976). Patients undergoing immunosuppressive therapy following transplants, are usually severely granulocytopoenic (Peterson, 1980). Such patients have increased susceptibility to P. aeruginosa pneumonia and bacteraemia.

The other major components of the systemic host defences which play a role in controlling P. aeruginosa invasion are the humoral immune system, the complement system and the cell-mediated immune system (CMI). Humoral immunity and CMI both involve complex interactions between macrophages and lymphocytes. The ultimate humoral immune response is antibody production by plasma cells

derived from B-lymphocytes, whereas CMI involves activation and transformation of T-lymphocytes in response to antigen (Peterson, 1980). The transformed T-cells secrete soluble mediators of immune reactivity (lymphokines), which in turn stimulate the activity of macrophages (Peterson, 1980).

The principle functional antibodies against P. aeruginosa appear to be opsonins of the IgG and IgM immunoglobulin classes (Young, 1974). Ninety percent of normal humans over the age of two years have IgM antibodies in their sera directed against P. aeruginosa (Gaines & Landy, 1955). IgM opsonizing antibody has an obligatory requirement for complement, as phagocytes do not have IgM receptors (Young, 1974), whereas IgG opsonization is potentiated by complement but can proceed without it (Young, 1974). Opsonizing antibodies are generally directed against the lipopolysaccharide (LPS) component of the P. aeruginosa outer membrane, which is responsible for the resistance of this organism to phagocytosis in the absence of opsonization (Young, 1972).

The other principle effector mechanism of humoral immunity is complement-dependent bacteriolysis (Høiby, 1979). However, since 91% of invasive P. aeruginosa strains are serum resistant (Young & Armstrong, 1972; Young, 1974), this mechanism is probably of little significance in controlling acute P. aeruginosa infections.

It is difficult to assess the role of antibodies in susceptibility to P. aeruginosa pneumonia due to the interrelated roles of the phagocyte, antibody and complement systems. However, McCall et al. (1973) reported three patients with IgM deficiency who

had defective opsonic activity against P., aeruginosa resulting in severe pulmonary infection. Complement-mediated and PMN function was normal in these patients.

The diagnostic and prognostic significance of antibodies to P. aeruginosa in acute infections is that patients with high antibody titres have a better prognosis than patients without such antibodies (Høiby, 1979), e.g. in the case of the leucopoenic dog model of experimental pneumonia (Kazmierowski et al, 1977). Antibody production is also important for the neutralization of the extracellular bacterial products which appear to be primarily responsible for the tissue damaging capabilities of P. aeruginosa (Peterson, 1980).

The importance of CMI in the control of P. aeruginosa invasion has not been extensively studied (Aduan & Reynolds, 1979). In fact, the incidence of P. aeruginosa infection is low in patients with Hodgkin's disease, which is associated with defective T-lymphocyte function (Aduan & Reynolds, 1979). However, Porwoll et al. (1983) demonstrated that T-lymphocytes from normal adults proliferate in vitro in the presence of P. aeruginosa suggesting that such a response may be important in controlling against infection.

Chronic pulmonary infection in CF patients

Although the characteristics of the P. aeruginosa strains associated with chronic pulmonary infections in CF patients will be discussed in detail in the next chapter, two of these characteristics are worth mentioning in this context because of

their relevance to studies of the host defence mechanisms involved in this particular infection. Firstly, P. aeruginosa isolates from the sputa of CF patients are frequently mucoid (Doggett et al, 1964; Doggett et al, 1966; Doggett, 1969; Høiby, 1975). Mucoid P. aeruginosa were first reported in 1927 (Sonnenschein, 1927) but until the 1960s were considered a rarity. Indeed, isolation rates for mucoid strains from sources other than CF patients range from 0.8% (Cétin et al, 1965) to 3% (Høiby, 1975), whereas 70-80% of P. aeruginosa isolates from CF patients are mucoid (Doggett, 1969; Høiby, 1975). Patients suffering from chronic lung disease with bronchiectasis, but not CF, are sometimes colonized with P. aeruginosa, and in one study, mucoid strains accounted for 10% of isolates associated with this condition (Rivera & Nicotra, 1982).

The origin of these mucoid strains will be discussed in the following chapter, however it is generally believed that the presence of mucoid P. aeruginosa in the respiratory tract of a CF patient is associated with chronic infection and a poor prognosis, whereas non-mucoid isolates merely reflect harmless colonization. Høiby (1974) has shown that the presence of mucoid P. aeruginosa in the sputum is associated with a significantly greater number of precipitating antibodies in the serum, directed against P. aeruginosa cellular components, than when non-mucoid strains only are present. More recently, Henry et al. (1982) reported that CF patients harbouring mucoid P. aeruginosa have significantly lower Schwachman scores (indicating a poorer clinical condition) than patients carrying non-mucoids, and they concluded from this that

mucoid P. aeruginosa are true pathogens, whereas non-mucoids are of little significance.

The second unusual characteristic associated with P. aeruginosa isolates from CF patients is serum sensitivity. As previously mentioned, Young (1974) noted that only 9% of invasive P. aeruginosa isolates were sensitive to the bactericidal action of normal human serum, and likewise, 21% of "colonizing" strains, i.e. isolated from patients but not associated with infection. However, Thomassen & Demko (1981) observed that 46% of P. aeruginosa strains from CF patients were sensitive to normal human serum, and Penketh et al. (1983a) reported 53% serum sensitivity amongst their CF P. aeruginosa isolates.

Apart from the increased incidence of respiratory tract infections, CF patients are no more susceptible to infections than normal children of comparable age (Schiøtz, 1981). Consequently, there appears to be no generalized immunodeficiency in these patients (Høiby et al, 1975). Unlike the acute invasive lung infections seen in immunocompromised individuals, systemic spread of P. aeruginosa is rarely encountered in CF patients (Huang & Doggett, 1979), suggesting that granulocyte function is normal. Studies relating to the humoral immune response in CF patients indicate that immunoglobulin levels in serum and sputum are normal or appropriately increased (Wallwork et al, 1974; Schiøtz et al, 1979a). Numbers of T and B lymphocytes are also normal (Høiby & Mathiesen, 1974). However, there is some evidence that IgG from CF patients may have reduced opsonic antibody function because of a molecular change in the Fc portion (Fick & Reynolds, 1983). There

may also be a structural abnormality in secretory IgA (Wallwork et al, 1974).

There are a number of reports in the literature of "blocking factors" in CF serum and sputum which protect serum sensitive strains from the bactericidal activity of normal serum. Thomassen & Demko (1981) reported that 5/12 patients in poor clinical condition harboured P. aeruginosa strains which were sensitive to normal serum but resistant to their own, indicating the presence of a "blocking factor" which was specific for their own strain. The authors considered that this factor may be IgG. Penketh et al. (1983a) identified a titratable "blocking factor" in the serum of 6/61 CF patients. This component was indeed present in the IgG fraction, interfered with the bactericidal activity of normal serum on the autologous P. aeruginosa strain but could not be absorbed out by that strain. Hence, it was suggested that this "blocking factor" may be directed against naturally occurring IgM. In this study there was no correlation between "blocking factor" and clinical condition or duration of P. aeruginosa colonization. Schiller & Millard (1983) demonstrated the presence of "protective activity" in 18/19 CF sputa, which was effective for both autologous or heterologous P. aeruginosa strains. This activity could be absorbed out, was not related to clinical condition, and considered to be IgA. A second, non-adsorbable "protective/blocking factor" was identified in this study and this was thought to be a bacterial product.

A specific IgG "blocking factor" has also been identified in the sera of two non-CF patients suffering from chronic P. aeruginosa

infections - one a respiratory tract infection and the other a urinary tract infection (Guttman & Waisbren, 1975). These authors proposed that some patients with chronic infection develop a population of antibody which inhibits and/or replaces the normal bactericidal IgM population. They further pointed out that IgG only manifests itself as a blocking antibody when the structure of the infecting organism is unfavourable for IgG-mediated complement activation, and that the density and distribution of antigen receptor sites on the bacterial surface may influence the effect of antibacterial IgG. However, the authors also indicated that it was not possible to determine whether the presence of blocking antibodies was the cause or effect of the chronic infection.

The possibility that CMI may be defective in CF patients has received some attention. Sorensen and colleagues (Sorensen et al, 1977; Sorensen et al, 1978; Sorensen et al, 1979) noted a significant specific incapacity of lymphocytes from severely affected patients to respond to P. aeruginosa and certain other Gram negatives. There was some indication that both cellular and plasma factors are involved in this dysfunction and in two patients, the capacity to respond recovered after vigorous antibiotic therapy.

In another study, Gibbons et al. (1976) demonstrated that severely ill CF patients had greatest impairment of CMI, but this could be reversed by steroid treatment.

Numerous studies have implicated a central role for the systemic host defences in the pathogenesis of chronic pulmonary infection in CF patients. Immune complexes have been demonstrated

in the sera and sputa of such patients (Schiøtz et al, 1978; Moss & Lewison 1980; Høiby & Schiøtz, 1982) and the role of these immune complexes in complement activation leading to tissue damage is well documented (Schiøtz et al, 1979a; Skov, 1980; Høiby & Schiøtz, 1982). It has also been noted that P. aeruginosa is capable of evoking an IgE response in some CF patients which could result in an allergic reaction (Pitcher-Wilmott et al, 1982).

CHAPTER III

BACTERIAL FACTORS ASSOCIATED WITH THE PATHOGENESIS OF P. AERUGINOSA RESPIRATORY TRACT INFECTIONS

Pathogenicity is the capacity of a microorganism to produce disease in a susceptible host. In order to do so, the pathogen must be able to enter the host, multiply and damage host tissue, and either resist, or not stimulate the host defences (Smith, 1977). The microbial factors responsible for these processes are the determinants of pathogenicity or the degree of pathogenicity (virulence), and may be cell-associated or extracellular.

There are several potential problems associated with the study of microbial pathogenicity. Firstly, virulence is only manifested *in vivo*, and bacteria grown *in vitro* may not express all their virulence determinants (Smith, 1977), hence meaningful studies on the role of particular virulence determinants in pathogenesis require experimental models which mimic as closely as possible the susceptible host and disease in question. The second problem is that the virulence determinants relevent to one disease are not always important in another - an important factor when considering the pathogenicity of *P. aeruginosa* which is capable of causing many different kinds of infections at many different sites. Thirdly, the fact that *P. aeruginosa* is an opportunist pathogen poses the problem of deciding which aspects of the disease are the result of host factors and which are mediated by the organism.

In certain cases, microbial products may not directly cause tissue damage, however, the mere presence of the bacteria, e.g. on the surface of a mucous membrane, may be sufficient to stimulate host-mediated tissue damage. Therefore, the microbial characteristics associated with colonization may have a pathogenic role.

There are a number of cellular and extracellular factors which have been linked with the pathogenicity of P. aeruginosa and these are summarized in Table 1. The main purpose of this chapter is to examine the role of these virulence determinants in acute and chronic respiratory infections, and also to consider the pathogenic significance of certain unusual characteristics which have been identified in P. aeruginosa isolates from chronic respiratory infections, particularly in CF patients.

Unusual characteristics associated with P. aeruginosa from chronic pulmonary infections

As mentioned previously, P. aeruginosa isolates from CF patients and patients with bronchiectasis are frequently mucoid, whereas isolates from other clinical sources, and from the environment, rarely have this characteristic (Doggett, 1969; Høiby, 1975; Rivera & Nicotra, 1982). Early biochemical studies on the exopolysaccharide produced by a mucoid P. aeruginosa isolate from the sputum of a CF patient, revealed that the substance was a polyuronide with properties similar to alginic acid (Linker & Jones, 1964). Subsequently, it was shown that mucoid strains from CF and non-CF sources produced an acetylated copolymer of β-D-mannuronic

TABLE 1 Cellular and extracellular products of *P. aeruginosa* thought to be associated with pathogenicity.

Product	Pathogenic role	Reference
Pili	Associated with adherence to buccal cells and acid injured ciliated respiratory epithelium	Woods *et al*, 1983 Ramphal *et al*, 1984
Lipopolysaccharide	Endotoxic and anti-phagocytic properties	Greer & Milazzo, 1976 Cryz *et al*, 1984
Flagella	Associated with invasion of burned tissue	McManus *et al*, 1980 Montie *et al*, 1982
Leucocidin	Specific cytotoxic action on human leucocytes	Scharmann, 1976
Slime (glycolipo-protein fraction)	Causes leucopoenia in mice	Bartell, 1983
Alginate	Associated with adherence to ciliated respiratory epithelium, antiphago-cytic properties and reduced pulmonary clearance	Baker & Marcus, 1982 Ruhen *et al*, 1980 Baltimore & Mitchell 1980 Govan *et al*, 1983
Exotoxin A	Inhibits eucaryotic protein synthesis	Iglewski *et al*, 1977
Exoenzyme S	Inhibits eucaryotic protein synthesis	Iglewski *et al*, 1978
Proteases:		
Elastase	Degrades plasma proteins and causes tissue damage at various sites	Wretlind & Pavlovskis, 1983 Schultz & Miller, 1974
Alkaline Protease	Associated with local tissue damage	Liu, 1979

TABLE 1 (cont.)

Product	Pathogenic role	Reference
Haemolysins:		
Phospholipase C	May degrade pulmonary surfactant. Toxic for alveolar macrophages	Liu, 1974 Al-Dujaili, 1976
Glycolipid	Solubilizes phospho-lipids thus enhancing the activity of phospholipase C	Kurioka & Liu, 1967
Phenazine pigment	Inhibits ciliary movement	Reimer *et al*, 1980

acid and its C-5 epimer α-L-guluronic acid with structure and properties remarkably similar to algal alginate (Carlson & Matthews, 1966; Linker & Jones, 1966). Apart from mutants of the closely related Pseudomonas species *P. putida*, *P. fluorescens* and *P. mendocina* (Govan *et al*, 1981), the only other bacterial source of this kind of exopolysaccharide is *Azotobacter vinelandii* (Gorin & Spencer, 1966).

It is important at this point to make a distinction between the alginate-like exopolysaccharide produced by mucoid *P. aeruginosa* strains, and slime, which is a loosely defined material with variable composition depending upon the strain, cultural conditions and method of analysis. The term 'mucoid' is restricted to those strains producing the characteristic colonial type 5 of Phillips (Phillips, 1969) within 24h on agar media, whereas many classic non-mucoid strains (Phillips colonial types 1 to 4) produce viscid, slimy colonies and broth cultures, particularly when incubation is prolonged, and in media with a high carbon content (Haynes, 1951).

Evidence for the emergence and establishment of mucoid *P. aeruginosa* strains in CF patients was first provided by Doggett *et al* (1966) who observed that in individual CF patients, initial colonization was by a non-mucoid strain, but during the course of infection, mucoid isolates gradually emerged and eventually came to predominate. However, this idea was not universally accepted as Zierdt & Williams (1975) believed that "the mucoid *P. aeruginosa* strain is probably spread from patient to patient, rather than acquiring its mucoid characteristic *de novo* in the CF patient.

The second unusual characteristic associated with P. aeruginosa isolates from chronic pulmonary infections is antibiotic hypersensitivity. Whereas the minimal inhibitory concentration (MIC) of carbenicillin for P. aeruginosa is generally 25-50μg ml^{-1} (Knudsen et al, 1967), May & Ingold (1972) reported that 35% of strains isolated from sputum (from CF patients, chronic bronchitics or patients with bronchiectasis), were sensitive to 6μg ml^{-1} carbenicillin, and some had MIC's as low as 0.7μg ml^{-1}.

In a later study, Berche et al (1979) compared 47 mucoid with 71 non-mucoid clinical isolates on the basis of sensitivities to 18 antibiotics and concluded that both groups could be divided into two distinct classes on this basis. Class A contained strains significantly more resistant to antibiotics such as the aminoglycosides and tetracycline than those strains allocated to Class B. These workers did not distinguish the strains on the basis of their origin as they were attempting to show a significant difference between the mucoid and non-mucoid isolates. This they failed to do.

Another group of workers (Seale et al, 1979), in an examination of serotypes and antibiotic susceptibilities of P. aeruginosa isolates from single CF sputum specimens, found that 20% of sputa contained isolates heterogeneous in their susceptibilities to the antibiotics carbenicillin, chloramphenicol, gentamicin, cotrimoxazole, streptomycin and tetracycline. Sensitivities were assessed using antibiotic disks and again there was no association between antibiogram and expression of the mucoid character.

The percentage of patients harbouring multiple P. aeruginosa strains onthe basis of antibiotic sensitivities as determined by MIC's was 51% according to a similar survey by Thomassen et al (1979). Despite this finding, only one serotype was generally present in a single patient and fromthis it was concluded that a patient with CF may be infected with one strain of P. aeruginosa that is capable of expressing many variant characteristics.

The surveys of Seale et al (1979) and Thomassen et al (1979) did not specifically report P. aeruginosa isolates which were unusually susceptible to antibiotics, however a study carried out in our own laboratory (Irvin et al, 1981) reported a number of P. aeruginosa isolates from CF patients that were unusually sensitive (hypersensitive) to a range of antibiotics including the β-lactams carbenicillin, azlocillin and methicillin, and to the unrelated antibiotic, trimethoprim. The carbenicillin MIC's of these strains were $<1\mu g\ ml^{-1}$, and thus comparable with those reported by May & Ingold (1972). In addition, we reported the isolation of a "normal" and hypersensitive strain (492a and 492c) from the same sputum specimen. These two strains were of the same pyocin type, suggesting that the hypersensitive strain may have emerged in vivo.

Various workers have reported that a significant proportion of P. aeruginosa isolates from CF patients are sensitive to normal human serum (Thomassen & Demko, 1981; Penketh et al, 1983b). In a survey of 109 CF isolates, 87 sputum isolates from non-CF patients and 118 isolates from other clinical sources, Penketh et al (1983a) confirmed that 53.2% of the CF isolates were serum sensitive,

compared with 13.8% of non-CF sputum isolates and 11.1% of isolates from other clinical sources. In addition, they observed that 64% of the CF strains could not be serotyped due to the loss of the O-type reaction, and 50.5% were agglutinated by more than one of the typing sera, i.e. were polyagglutinable (PA). Amongst the non-CF sputum isolates they noted 17% were PA, whereas only 10.2% of the non-respiratory isolates expressed this characteristic. Although there was a close correlation between loss of the O-type reaction, PA and serum sensitivity, some "intermediate" strains were identified with some, but not all of these features.

Schiller & Hatch (1983) carried out a survey of P. aeruginosa isolates from various clinical sites to determine whether there was any relationship between certain microbial characteristics, including serum sensitivity and serotype, and the source of the particular strain. These workers confirmed that CF isolates were more likely to be serum sensitive than non-CF isolates (26/30 CF isolates were serum sensitive compared with 4/22 sputum isolates from non-CF patients and 3/41 from wounds, blood, urine and burns). They also observed that nine of the CF isolates which were serotyped were PA (it is not clear whether all 30 CF strains were in fact serotyped).

The structural basis of serum sensitivity and PA in clinical P. aeruginosa isolates from CF patients has been examined by Hancock et al (1983). Results of this study suggested that PA strains were agglutinated by antibody in the typing serum directed at non-serotype determinants, rather than expressing multiple immunotype

antigens. All the PA strains were serum sensitive, however, not all the typable strains were serum resistant. Hancock et al suggested that the structural basis for PA and serum sensitivity in P. aeruginosa isolates from CF patients, is a deficiency in the smooth O-antigen-containing LPS, although a number of the typable serum resistant strains were also apparently deficient in this component of the outer membrane in this study.

Meadow et al (1984), using PA mutants of P. aeruginosa strain PAC1R, have confirmed that changes in the LPS composition of such mutants, expose other outer membrane components, e.g. outer membrane proteins, and rough core components, which are responsible for the cross-reactivity with heterologous O-antisera. The nature of the LPS changes identified in their mutants ranged from complete lack of O-antigenic components to altered mobility of the high molecular weight LPS fraction on Sephadex G75.

Bacterial attachment and initial colonization of the respiratory tract

An important event in the establishment of both acute and chronic respiratory tract infections is the initial bacterial colonization of the upper respiratory tract. Although host factors are largely responsible for allowing P. aeruginosa "a foot in the door", it is important to discover which bacterial factors are important in the initiation of colonization, especially if this step is to be prevented.

Adherence of P. aeruginosa to human buccal epithelial cells has been shown to be mediated by pili, on the experimental basis that purified pili, when preincubated with buccal cells, decrease the adherence of intact organisms (Woods et al, 1983). However, it is not clear whether attachment to buccal cells in vivo is an important step in P. aeruginosa colonization of the respiratory tract. The ability to adhere to ciliated epithelium may be more relevant, and in one study, Niederman et al (1983) compared bacterial adherence to human buccal cells and ciliated epithelial cells from the nose and trachea. The results of this study indicated that P. aeruginosa adheres better to ciliated epithelium than squamous epithelium in vitro, and that the mechanisms involved in such adherence are probably different for the two sites. In addition, in normal subjects, bacterial adherence at one site did not correlate with adherence at another.

Two different groups have developed experimental models for examining the adherence of P. aeruginosa to tracheal epithelium Ramphal and coworkers exposed mouse tracheas to 0.1N HCl for 15 mins, after which they were cut into sections and placed in petri dishes for adherence studies. Ramphal & Pyle (1983a) showed that adherence to acid injured tracheal cells was a phenomenon limited to P. aeruginosa (both mucoid and non-mucoid strains), and did not occur with Escherichia coli or Klebsiella pneumoniae. They also showed that mucoid P. aeruginosa adhered to mucin strands of uninjured control tracheas. In another study (Ramphal & Pyle, 1983b) these workers showed that mucin and sialic acid inhibited the adherence of mucoid and non-mucoid strains of P. aeruginosa to acid

injured tracheal cells. The affinity of P. aeruginosa (both mucoid and non-mucoid) for tracheobronchial mucin has recently been confirmed using an in vitro assay (Vishwanath & Ramphal, 1984).

The role of pili in the adherence of P. aeruginosa to acid injured tracheal epithelium has also been examined by Ramphal et al (1984) in the absence of mucin. In this study, pili incubated with tracheal cells inhibited the adherence of a non-mucoid strain in a dose-dependent manner. However, pili did not appear to be the final mediators of adherence of mucoid strains, suggesting that there are differences in the receptors and mechanisms of adhesion for mucoid and non-mucoid strains in this model.

Baker & Tao (1982) have reported an alternative tracheal culture model using hamster tracheal sections incubated under tissue culture conditions whereby ciliary activity is maintained for up to two days. Using this model, Baker & Marcus (1982) compared the adherence of mucoid and non-mucoid clinical P. aeruginosa isolates to the tracheal epithelium. The mucoid organisms were seen adhering to the cilia in clusters, embedded in an extracellular matrix, whereas the non-mucoid organisms generally adhered singly. In addition, the mucoid isolates adhered more rapidly than the non-mucoid isolates, indicating a more efficient mechanism of attachment.

Long-term bacterial survival in therespiratory tract

Initial colonization is an important step in the establishment of both acute and chronic respiratory tract infections, and probably

involves similar mechanisms in both cases. However, the factors necessary for bacterial survival and multiplication thereafter are largely dependent on the state of the host defences. As discussed in the previous chapter, individuals who succumb to acute P. aeruginosa pneumonia are usually neutropoenic and thus incapable of resisting bacterial invasion. The breakdown of host tissue releases nutrient for bacterial growth (Cicmanec & Holder, 1979), hence the bacterial factors responsible for multiplication in host tissue and causing tissue damage are probably similar.

In contrast, CF patients and other individuals susceptible to chronic P. aeruginosa infections are not as severely compromised and as a result, a host-parasite relationship develops between bacterium and host. In CF patients, the relationship between host and opportunist is very finely balanced.

Most patients tolerate heavy P. aeruginosa colonization for long periods of time without morbidity or decline in lung function, however, small changes in bacterial numbers may be sufficient to tip the scales, as one of the features of chronic infection in these patients is the intermittent development of acute exacerbations, i.e. malaise, weight loss and symptoms of acute bronchitis associated with increased bacterial counts in sputum (Geddes, 1982).

Experimental evidence suggests that bacterial alginate may be important for long term survival of mucoid P. aeruginosa in the lower respiratory tract, as in addition to its adhesive properties, alginate has been shown to inhibit bacterial opsonization by rabbit antiserum in vitro (Baltimore & Mitchell, 1980), and to inhibit

phagocytosis by alveolar macrophages cultured from rats and guinea pigs (Ruhen et al, 1980). Alginate may also present a physical and ionic barrier to the penetration of aminoglycoside antibiotics (Slack & Nichols, 1981). Costerton's group have reported that mucoid P. aeruginosa could be seen as encapsulated microcolonies in the alveoli of post-mortem lung tissue from a CF patient (Lam et al, 1980). This is understandable when one considers the physical properties of P. aeruginosa alginate, in particular the capacity to form a gel in 3mM Ca^{2+} - a level which one would expect to find in pulmonary secretions (Govan et al, 1983). Surprisingly perhaps, Blackwood & Pennington (1981) could show no difference between mucoid and non-mucoid strains in terms of pulmonary clearance by guinea pigs. However, these workers used washed bacterial cells and it should also be remembered that the mucociliary clearance mechanism in a normal guinea pig could not be compared with that of a CF patient (or bronchiectic). The use of unwashed cells in a similar experimental model (but using rats instead of guinea pigs), demonstrated that a mucoid strain was cleared less efficiently than an isogenic non-mucoid strain (Govan et al, 1983).

The role of other surface components in the survival of P. aeruginosa in the lung has not been systematically studied. It is well established that CF patients chronically infected with P. aeruginosa have high titres of antibodies directed against O-antigens in both serum and sputum (Høiby, 1979). Thus bacteria lacking the O-antigen may be resistant to opsonization by these antibodies, in other words, a defective LPS would provide a

selective advantage in this situation and could be considered a virulence determinant.

While the structural basis of antibiotic hypersensitivity in clinical P. aeruginosa isolates has not yet been determined, antibiotic hypersensitive strains of Neisseria gonorrhoeae are frequently isolated from cases of gonorrhoea (Eisenstein & Sparling, 1978). The outer membranes of these strains have increased permeability, and this is thought to aid colonization of the mucosal surfaces (Lysko & Morse, 1981).

Various extracellular virulence determinants may be important for the long-term survival of P. aeruginosa in the lung. Antibodies directed against alkaline protease, elastase and exotoxin A have been detected in the serum of CF patients (Klinger et al, 1978; Döring, et al, 1983; Döring & Høiby, 1983; Cukor et al, 1983) indicating that these enzymes are produced in vivo by the colonizing/infecting strains. Proteases may potentiate bacterial survival in vivo through the inactivation of complement factors (Schultz & Miller, 1974) and cleavage of IgG (Fick et al, 1982).

Jagger et al (1983) have reported that 65% of 380 P. aeruginosa isolates from CF patients had elastase activity and 64% had alkaline protease activity. They also noted that strains from colonized patients were more proteolytic than those from chronically infected patients, suggesting that protease activity may be selected against in this situation.

Cash et al (1979) have developed a rat model of chronic respiratory infection with P. aeruginosa, where bacteria

encapsulated in agar beads are instilled into the lungs intratracheally. The lesions associated with this experimental infection are similar to those described in patients with non-bacteraemic P. aeruginosa pneumonia (Renner et al, 1972; Tillotson & Lerner, 1968) and CF patients chronically infected with P. aeruginosa (Bedrossian et al, 1976), i.e. goblet cell hyperplasia, focal areas of necrosis, and acute and chronic inflammatory infiltrate.

One research group has attempted to develop an animal model of CF by treating rats with reserpine on a daily basis for 14 days (Thompson et al, 1976; Wood & Martinez, 1977; Perlmutter & Martinez, 1978). The pulmonary secretions of chronically reserpinized rats showed some of the alterations associated with CF, however, the treatment gave rise to so many diverse side effects that the model is no longer considered to mimic CF. To my knowledge, there are no published reports of bacteriological studies using this model.

Various groups have used the Cash model of chronic respiratory infection to examine the role of particular P. aeruginosa virulence determinants on bacterial survival and ability to cause pathological lesions in the lung (Woods et al, 1982; Sokol & Woods, 1984; Cash et al, 1983). It is generally agreed that neither exotoxin A nor elastase contribute significantly to bacterial survival in the rat lung.

According to Al-Dujaili (1976), the P. aeruginosa haemolysins may contribute to bacterial survival in the lung due to its toxic activity against alveolar macrophages.

Lung damage in acute and chronic infections

Fetzer et al (1967) examined post-mortem lung tissue from seven patients who had died from bacteraemic P. aeruginosa pneumonia and noted two types of gross lesions:- (i) poorly defined, haemorrhagic, nodular, indurated areas, occasionally with a small central necrotic locus, (ii) firm, yellow-brown, necrotic nodules with slightly raised, well-demarcated, lobulated borders. These workers commented that the second type of lesion was quite unlike that seen in other forms of bronchopneumonia. They also noted that bacteria were almost invariably found adjacent to capillaries and not in the walls of arteries and veins suggesting that colonization of the capillaries may provide a route for bacterial dissemination.

Gray & Kreger (1979) have reported that lesions very similar to those described above can be elicited in the lungs of rabbits by the experimental administration of P. aeruginosa proteases. Using a guinea pig model of acute P. aeruginosa pneumonia, Blackwood et al (1983) evaluated the roles of exotoxin A and elastase as virulence factors in this kind of infection. They concluded that elastase, but not exotoxin A had a significant pathogenic role.

P. aeruginosa has been shown to be the cause of epidemics of haemorrhagic pneumonia in mink (Shimizu et al, 1974), and using this infection as an experimental model, Homma et al (1983) have evaluated a multicomponent vaccine, consisting of the common protective P. aeruginosa antigen (OEP) and toxoids of protease and elastase, for its ability to prevent an epidemic of haemorrhagic pneumonia in a mink population. These workers showed that the

multicomponent vaccine was significantly more effective in this respect than a single component vaccine (containing OEP only), suggesting that protease and elastase contribute to the virulence of the P. aeruginosa strain in this infection, whether in terms of bacterial invasion or survival in the lung.

Using the rat model of chronic respiratory infection, Cash et al (1983) have reported that histopathological changes were noted when exotoxin A and protease, alone or in combination, were instilled. Similar conclusions have been drawn by Woods et al (1972) and Sokol & Woods (1984). In addition, Cash et al (1983) noted that the continued presence of P. aeruginosa and its products served to maintain a chronic inflammatory response. These workers postulated that the accumulation of PMN's at the site of the lesion is induced and maintained by LPS due to its chemoattractant properties.

In the hamster tracheal organ culture model, Baker (1982) has shown the exotoxin A and elastase, but not alkaline protease, contribute to the destruction of ciliated epithelium.

It has been postulated that haemolysins produced during P. aeruginosa lung infections may degrade surfactants leading to atelectasis and other pathological alterations (Liu, 1979).

It would be reasonable to conclude, from this review of the literature, that the pathogenicity of P. aeruginosa in the lung involves the expression and interaction of various different factors, the relative importance of which vary according to the type

of infection or experimental model used to study them. Whereas the factors likely to cause tissue damage in acute infection are characteristic of the majority of clinical isolates of P. aeruginosa, the unusual characteristics associated with long-term colonization and chronic infection of the lung are a reflection of the adaptability of this complex microbe.

CHAPTER IV

GENETICS AND *P. AERUGINOSA* PATHOGENICITY

The role of genetics in the study of P. aeruginosa pathogenicity

In recent years, the role of genetics in the study of microbial pathogenicity has become increasingly important, due to the rapid developments in technology for the genetic manipulation of micro-organisms. In the case of *P. aeruginosa*, classical genetic techniques (e.g. the isolation of particular mutants), in combination with animal models, have been used to assess the role of exotoxin A, elastase and alkaline protease in corneal infections (Ohman *et al*, 1980; Howe & Iglewski, 1984), the role of proteases in burn infections (Pavlovskis & Wretlind, 1979), and the role of exotoxin A and elastase in chronic lung infections (Woods *et al*, 1982; Sokol & Woods, 1984) and acute lung infections (Blackwood *et al*, 1983). In addition, the isolation of a *P. aeruginosa* mutant producing a non-toxic, immunologically cross-reactive exotoxin A protein (Cryz *et al*, 1980) has recently led to the development of a toxoid of exotoxin A with potential as a vaccine component (Cryz & Iglewski, 1983).

The genetic basis, and particularly the regulation, of *P. aeruginosa* virulence determinants, e.g. exotoxin A, phospholipase C and elastase, has been the subject of considerable interest and research. The majority of the published reports have described the use of classical genetic techniques, particularly R68.45 mediated conjugation for the mapping of genetic loci associated with these

extracellular products. Recently, workers such as Coleman et al (1983) and Vasil et al (1982) have examined the expression of a gene encoding phospholipase C through the use of recombinant DNA technology (cloning).

Because of the important and varied role of genetics in the analysis of P. aeruginosa pathogenicity, I will briefly outline the history of P. aeruginosa genetics, including the development of techniques for the genetic manipulation and analysis of the organism and then give examples of their use in the study of virulence.

The genetics of P. aeruginosa

The isolation of auxotrophic mutants in P. aeruginosa was first described in 1955 by Loutit (Loutit, 1955) and in the same year, a system of genetic recombination, using conjugation, was described by Holloway (Holloway, 1955). The ready availability of P. aeruginosa bacteriophages from sewage and from clinical isolates of P. aeruginosa, led to the development of a transductional system (Holloway et al, 1960), and genetic mapping studies ensued. The P. aeruginosa strain chosen for detailed genetic analysis, originally designated Strain 1 (Holloway, 1955), and now known as strain PAO, was isolated from an infected wound in an Australian hospitalized patient. A second strain, designated PAT (isolated fromroutine clinical material in South Africa by Don & van den Ende, 1950) and the source of the "original" P. aeruginosa sex factor, FP2, has also been subjected to genetic analysis (Watson & Holloway, 1978), but not on the same scale as strain PAO.

For over 20 years detailed mapping of the PAO chromosome was hampered by the inability to demonstrate genetic circularity. The reason for this was that FP2 and various other sex factors such as FP5 (Matsumoto & Tazaki, 1973) and FP39 (Pemberton & Holloway, 1973) apparently transferred the chromosome unidirectionally from a single origin. Consequently, the mapping of markers located beyond 40 mins from the origin of transfer was extremely inaccurate, due to the low recovery of recombinants. The chromosome was estimated to be 80-110 mins in length on the basis of a molecular mass of 2.1 x 10^9 daltons (Pemberton, 1974). Subsequently, another sex factor, FP110, has been shown to transfer the chromosome from an origin at about 27 mins (Royle & Holloway, 1980), but this has not been used in routine mapping studies due to the development of the alternative systems to be described.

In 1976, the isolation of a new sex factor, R68.45 (Haas & Holloway, 1976) represented a major advance in Pseudomonas genetics. R68.45 is a variant of the broad host range Inc P-1 plasmid R68 (Holloway & Richmond, 1973) which has chromosome mobilizing ability (Cma) in P. aeruginosa and various other Gram-negative organisms including P. putida (Martinez & Clarke, 1975), Escherichia coli and several species of Rhizobium (Beringer et al, 1978; Kondorosi et al, 1977; Megias et al, 1982). The molecular basis for Cma in R68.45 appears to be the duplication of a DNA segment occurring as a single copy in R68, thus giving rise to a transposable genetic element which is designated IS21 (Willetts et al, 1981). R68.45 possesses several interesting properties which make it an extremely useful genetic tool. (i) Chromosome mobilization occurs from multiple

origins and is non-polarized, hence chromosomal recombinants for most markers are formed at acceptable frequencies - about 10^{-4} per donor cell on solid media (Haas & Holloway, 1976; Haas & Holloway, 1978). (ii) In R68.45-mediated plate matings, most recombinants inherit short chromosome fragments (up to about 10 mins long) and the plasmid is lost from these recombinants during purification, hence R68.45 can be used like a "large" generalized transducing phage for the construction of strains (Haas & Holloway, 1978); (iii) It is possible to isolate derivatives of R68.45 which carry segments of chromosomal DNA (Holloway, 1978). Such plasmids, known as R primes (R'), are useful tools for the study of gene expression, as they allow the construction of partial diploid strains, and provide an intermediate step in the transfer of particular genes to a cloning vector.

Genetic circularity of the PAO chromosome was finally demonstrated in 1981 (Royle et al, 1981), and it was also shown that FP2-mediated chromsome transfer did not take place from a single origin as previously believed. Early mapping studies by Loutit (1969) had indicated that two FP2 linkage groups existed; a major one containing most markers mapped at that time, and a minor one containing four loci which were essentially unlinked to markers of the major linkage group. It is now clear that the minor linkage group is the consequence of chromosome transfer from one or several origins close to the major one, and in the opposite direction to that found for the major origin (Soldati et al, 1984).

In recent years, the discovery and subsequent utilization of transposons has revolutionized microbial genetics. Transposons are normal constituents of most bacterial genomes which may have evolved as nature's tools for genetic engineering because of their ability to rearrange other DNA sequences (Kleckner, 1981).

In P. aeruginosa, a strategy of transposon-facilitated recombination was described by Krishnapillai et al (1981), whereby several Tn1 insertions in the late chromsome region of PAO were obtained, from which the R plasmids R18 and R91-5 could mobilize the chromosome in a polarized fashion by virtue of Tn1 homology between chromosome and plasmid. Transposon insertion into the chromosome also provides a useful method for obtaining mutants, without the problem of multiple lesions, so often associated with chemical mutagenesis, particularly using nitrosoguanidine (NG) (Haas et al, 1984; Stapleton et al, 1984).

Another major development in the technology of P. aeruginosa genetics is the establishment of specialized host:vector systems for gene cloning (Wood et al, 1981; Bagdasarian, 1981; Bagdasarian & Timmis, 1981; Sakaguchi, 1982; Haas et al, 1984). Cloning provides a means of isolating and characterizing particular gene products as well as studying their expression.

The genetic basis of P. aeruginosa virulence determinants

Figure 2 is a diagram of the current P. aeruginosa chromosome map showing the locationsof markers relevent to this thesis. Included on the map are a number of loci associated with virulence

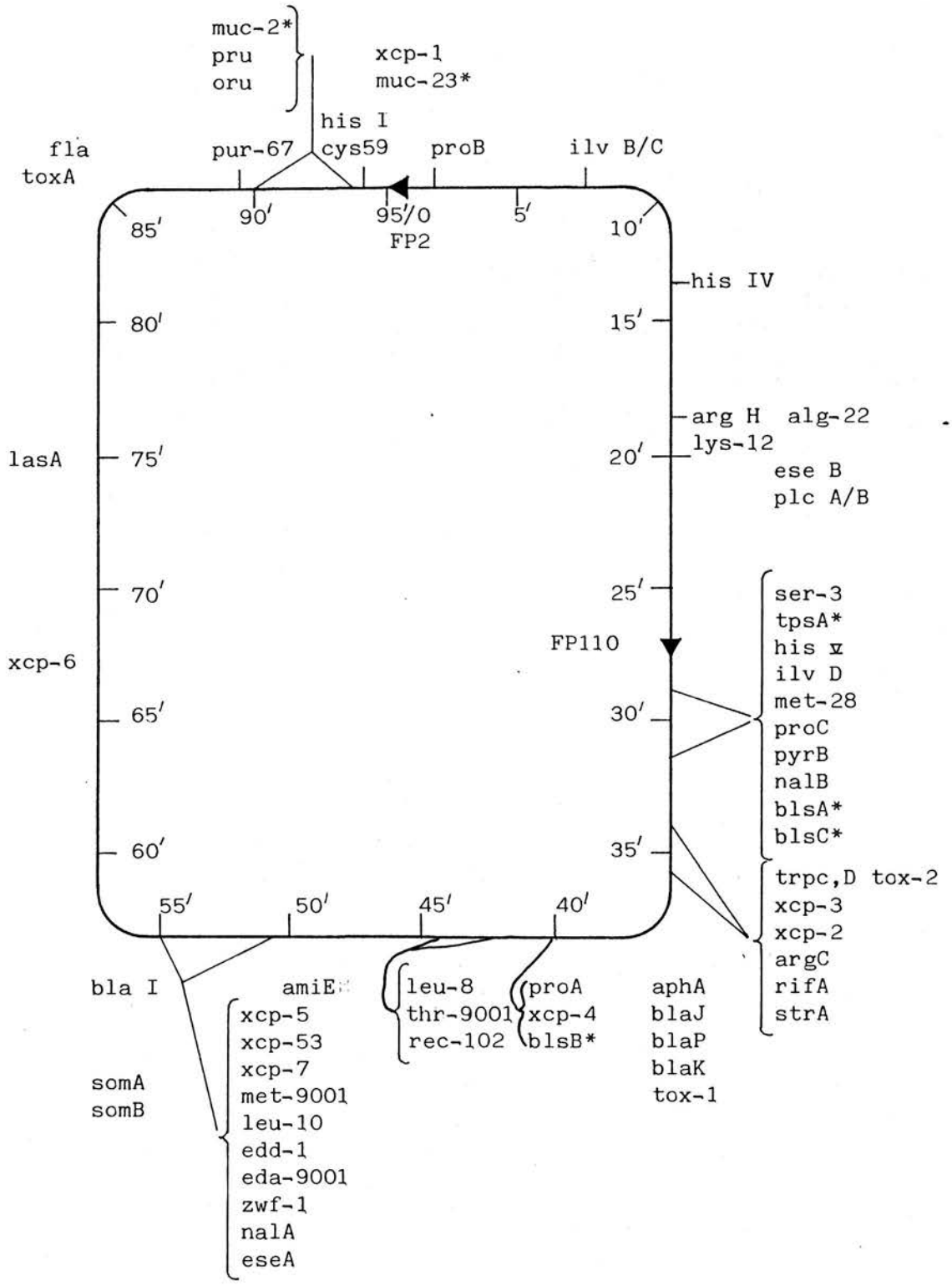


Figure 2. Chromosome map of P. aeruginosa PAO indicating the location of markers relevent to this thesis, based on the following references:- Holloway & Crockett (1982), Soldati et al (1984), Früh et al (1983), Kella & Haas

(1982), Hanne *et al* (1983), Howe *et al* (1983), Tsuda *et al* (1981), Okii *et al* (1983),Gray & Vasil (1981a), Roehl *et al* (1983), Wretlind & Pavlovskis (1984), Darzins & Chakrabarty (1984). Markers with asterisks were mapped during the course of this thesis. The following marker designations have been used: *alg*, instability of alginate production; *ami*, amidase; *aph*, aminoglycoside 3-phosphotransferase II; *arg*, arginine requirement; *bla*, B-lactamase production; *bls*, β-lactam hypersensitivity; *cys*, cysteine requirement; *eda*, 2-keto 3-deoxy 6-phosphogluconate aldolase; *edd*, 6-phosphogluconate dehydratase; *ese*, phage E79 resistance; *fla*, flagella; *his*, histidine requirement; *ilv*, isoleucine, valine requirement; *las*, elastase production; *leu*, leucine requirement; *lys*, lysine requirement; *met*, methionine requirement; *muc*, mucoid colonial type (alginate production); *nal*, resistance to nalidixic acid; *oru*, ornithine utilization; *plc*, phospholipase C; *pro*, proline requirement; *pru*, proline utilization; *pur*, purine requirement; *pyr*, pyrimidine requirement; *rec*, recombination ability; *ser*, serine requirement; *som*, somatic antigen; *str*, resistance to streptomycin; *thr*, threonine requirement; *tox*, exotoxin A production; *tps*, sensitivity to trimethoprim; *trp*, tryptophan requirement; *xcp*, extracellular protease production; *zwf*, glucose 6-phosphate dehydrogenase.

determinants, all of which are naturally expressed in PAO. The structural gene for exotoxin A (toxA) was identified following the isolation of a PAO mutant which produced an enzymatically inactive and non-toxic exotoxin protein, and the mapping of this locus was performed using R68.45 mediated plate matings (Hanne et al, 1983). Two additional loci tox-1 and tox-2 are associated with toxin production (Gray & Vasil, 1981a). Whereas tox-2 mutants are specifically deficient in exotoxin A, suggesting that this locus is involved in the regulation of synthesis, tox-1 mutants were also altered in the secretion or synthesis of other extracellular proteins (Gray & Vasil, 1981a). Several other loci associated with the regulation and release of extracellular proteins (including protease, elastase, staphylolytic enzyme and lipase) have also been mapped in PAO using R68.45 mediated conjugation (Wretlind & Pavlovskis, 1984). These loci, designated xcp appear to be distributed around the chromosome in small clusters. An xcp locus (xcp-7) in strain PAKS, thought to encode a proelastase activating enzyme, which also influences the release and/or activity of other extracellular proteins, has been transferred into PAO using R68.45 and mapped at 55' like xcp-5 and xcp-53 (Wretlind & Pavlovskis, 1983).

The structural gene for elastase (lasA) has been mapped at 75 mins, using both R68.45 mediated conjugation and Tn1 facilitiated recombination (Howe et al, 1983) following the isolation of a mutant specifically deficient in elastase production (Ohman et al, 1980).

Further studies on the regulation of extracellular virulence determinants in PAO have revealed that exotoxin A, alkaline protease and elastase yield are subject to independent iron regulation (Sokol et al, 1982). According to Bjorn et al (1979), growth of strains in vitro, in medium containing a high iron concentration(5.0μg ml^{-1}) causes an 85% decrease in exotoxin A yield as compared with strains grown in low iron (0.05μg ml^{-1}). In constrast, synthesis of phospholipase C and alkaline phosphatase in P. aeruginosa is subject to phosphate repression, and two closely linked chromosomal loci (plcA,B) associated with the coregulationof these enzymes have been identified and mapped (Berka et al, 1981; Gray & Vasil, 1981b; Gray et al, 1981; Gray et al, 1982). The structural gene for phospholipase C has not actually been mapped, but its expression has been examined through the use of cloning techniques. Vasil et al (1982) cloned the phospholipase C gene along with the structural genes of five other phosphate-repressible proteins, indicating the existence of a phosphate regulon. Coleman et al (1983) also cloned the structural gene for P. aeruginosa phospholipase C and noted that the gene product was a polypeptide of MW 78,000. Both of these studies succeeded in obtaining expression of the phospholipase C gene in E. coli where haemolytic activity was cell associated rather than extracellular, which is the case in P. aeruginosa.

Despite the fact that the regulation and synthesis of alkaline phosphatase and phospholipase C is different from exotoxin A and elastase they all seem to share a common release mechanism, whereas alkaline protease apparently has a a separate mechanism for regulation and release (Wretlind & Pavlovskis, 1984).

The other potential virulence determinants normally expressed in strain PAO are cellular components, i.e. pili, flagella and LPS. In an elegant study using R'*fla* derivatives of R68.45 for complementation analysis, Tsuda and colleagues (Tsuda *et al*, 1981) have identified two linked clusters of genes on the PAO chromosome (at approximately 85') associated with flagella synthesis and function. It was once thought that in general, genes controlling related functions in *P. aeruginosa* were non-clustered (Holloway, 1975). However, several other examples of gene clustering have now been demonstrated, particularly involving catabolic genes (Roehl *et al*, 1983). Evidence for a chromosomal location of the genetic determinants for polar pili has been reported (Bradley, 1980), however, the loci have not actually been mapped.

A systematic genetic study of LPS synthesis in *P. aeruginosa* has yet to be undertaken, however, several markers, potentially associated with this important outer membrane component have been mapped. The loci *eseA* and *eseB* are associated with resistance to the virulent phage E79 (Holloway *et al*, 1971). The receptor site for this phage in strain PAO has been shown to reside in the LPS (Jarrell & Kropinski, 1977), hence a mutation to phage resistance could mean loss or alteration of that receptor. Indeed, Jarrell & Kropinski (1977) confirmed that the LPS of E79^r PAO mutant lacked certain of the major constituents found in wild type LPS, and Meadow & Wells (1978) have since shown that in strain PAC, the E79 receptor is associated with the core fraction of the LPS. Matsumoto & Tazaki (1975) have mapped two loci (*somA* and *somB*) associated with heat-stable somatic antigens, by means of interstrain crosses. Although

biochemical analysis was not performed on the antigens in question, they were almost certainly LPS-associated, based on the work of Chester et al (1973) and Koval & Meadow (1975).

The genetic basis of characteristics not normally expressed in PAO

Certain characteristics expressed by clinical P. aeruginosa isolates are not normally expressed by strain PAO. There are several possible reasons for this. (i) PAO does not carry the necessary genetic information, whether chromosomal or plasmid-mediated, for the expression of the characteristic. In this case, to obtain a derivative of PAO capable of expressing the characteristic in question, one would first have to transfer the gene(s) responsible from an appropriate clinical strain into PAO. It would not be possible to isolate a PAO mutant with the desired properties. (ii) PAO carries the genetic information, but appropriate growth conditions are necessary for expression, i.e. the normal lack of expression is phenotypic rather than genotypic. (iii) PAO carries the necessary genetic information but this is normally repressed (genotypically). In this case, it would be possible to isolate mutants which expressed the characteristic.

Strain PAO does not normally express any of the unusual characteristics associated with isolates from chronic respiratory tract infections, i.e. alginate synthesis, antibiotic hypersensitivity, serum sensitivity and PA. Hence, the genetic analysis of these characteristics is not as straight-forward as, for example, elastase and exotoxin A production.

a) Alginate synthesis.

Despite some controversy, evidence suggested that mucoid P. aeruginosa strains arise in vivo from classic, non-mucoid strains (Doggett et al, 1966), as when the two forms are found together in the same specimen, they generally belong to the same serotype (Diaz et al, 1970) and pyocin type (Willams & Govan, 1973). However, in contrast to their emergence in vivo, when cultured in vitro, mucoid strains of P. aeruginosa tend to revert to the non-mucoid form (Zierdt & Schmidt, 1964).

Markowitz et al (1978) examined the possibility that alginate production by clinical mucoid P. aeruginosa isolates might be plasmid associated. Eighteen such strains were evaluated with respect to plasmid content and ability to maintain well characterized plasmids. Not surprisingly, no such association was observed.

The first report of in vitro isolation of mucoid variants was by Williamson (1956) who claimed that "mucoid colonies could be obtained in pure form by repeated transfer of the smooth state cells through 'eugon' broth enriched with 4% potassium gluconate, and the selective transfer of isolates possessing mucoid characteristics to a solid modification of Hayne's media". However, there was no indication whether such mucoid strains were alginate producers or merely copious producers of slime.

Martin (1973), during the course of phage typing, noted rings of mucoid growth around areas of phage lysis while the background

lawn remained non-mucoid. Subculture from these areas resulted in pure cultures of mucoid colonies which looked and behaved like clinical isolates. Martin believed that mucoid variants derived in this manner required the presence of phage in the lytic cycle for the continued expression of the mucoid character and used the term "pseudolysogeny" to describe this phenomenon.

An investigation of the influence of various substances on the stability of mucoid P. aeruginosa in vitro (Govan, 1975) showed that clinical and phage derived strains maintained their mucoid colonial form when serially subcultured on desoxycholate citrate agar (DCA), or in sodium deoxycholate broth.

In 1978, we published an alternative method for isolating mucoid variants in vitro without the use of phage (Govan & Fyfe, 1978), based on the observation that certain mucoid strains were slightly more resistant to some antibiotics than related non-mucod forms (Govan, 1976). In retrospect, this observation was somewhat fortuitious as the increased antibiotic resistance of mucoid strains became a subject of controversy (Markowitz et al, 1980; Irvin et al, 1981). The selective agent of choice in the isolation method was carbenicillin, though other β-lactams and tobramycin have also been used successfully.

The frequency of isolation of mucoid variants from strain PAO using this procedure was approximately 1 in 10^7 cells and this frequency could be increased following mutagenesis with ethylmethanesulphonate (EMS). Mucoid strains so derived do not require the continued presence of the antibiotic for expression of

the characteristic, and infra-red spectroscopy of the exopolysaccharide confirmed it to be an acetylated polymer of mannuronic and guluronic acids.

A modified version of the isolation procedure has since provided a means of isolating alginate-producing variants of P. putida, P. fluorescens and P. mendocina (Govan et al, 1981). Alginate biosynthesis in these species had not previously been reported.

From these results, we concluded that (i) P. aeruginosa strain PAO, and probably most other P. aeruginosa strains, have the necessary genetic information for the synthesis of alginate, but this is normally repressed, and (ii) expression of the genes involved in the alginate biosynthetic pathway occurs following a mutation, probably in a regulatory gene. Similarly, the instability of alginate synthesis under certain in vitro cultural conditions can be explained on the basis of mutation using a fluctuation test (Govan et al, 1979). The mucoid characteristic is generally less stable when the strain is grown in unshaken broth cultures than in shaken cultures due to the faster growth rate of non-mucoid revertants under these conditions (Govan et al, 1979). Boyce & Miller (1980, 1982) have further shown that the Fe^{2+} or Fe^{3+} concentration has a profound effect on the selection of non-mucoid revertants in unshaken cultures. Mucoid strains grown in medium containing 0.01mM iron were rapidly outgrown by non-mucoid revertants, whereas the revertants had less of a selective advantage in lower iron concentrations.

Two different approaches have so far been used for the analysis of loci associated with alginate synthesis in mucoid P. aeruginosa. Ohman & Chakrabarty (1981) have attempted to develop a genetic system in a clinical mucoid P. aeruginosa isolate and have obtained evidence for the chromosomal location of three mutations (alg) responsible for reversion to the non-mucoid form. Unfortunately it was not possible to relate the markers on their chromosomal map to the established PAO map. Our own genetic studies have utilized mucoid mutants of PAO, isolated according to Govan & Fyfe (1978) or following selection for resistance to the virulent phages E79 or M6 (Govan, 1975).

We based our approach on the early studies of Markovitz and colleagues relating to mucoid E. coli (reviewed in Markovitz, 1977), certain aspects of which I will summarize. Like P. aeruginosa, wild type E. coli K12 does not appear mucoid when grown on solid media at 37°C, however, unlike P. aeruginosa, this strain does produce an exopolysaccharide (colanic acid) when grown at 30°C. Mutant strains of E. coli K12, which produce mucoid growth at 37°C can be isolated - these mutants are derepressed for colanic acid synthesis. Early mapping studies revealed that at least two different chromosomal loci yielded mucoid strains (Markovitz, 1964), and these are presently designated capR (lon) and capS. A third locus capT, which yields mutants producing colanic acid on EMB-glucose medium (capR and capS mutants are non-mucoid on this medium) at 37°C was later identified (Markovitz, 1977).

The regulatory role of the capR gene was confirmed by partial diploid studies. Using an F' derivative carrying the capR$^+$ allele

(F'13), *capR*$^+$ was shown to be dominant over the *capR6* mutation, i.e. F'13*capR*$^+$/*capR6* heterogenotes were non-mucoid (Markovitz, 1964). A F'*capR6*/*capR*$^+$ strain was also shown to be non-mucoid (Markovitz & Rosenbaum, 1965). However, a second *capR* mutation, *capR9* was shown to behave differently fron *capR6*, i.e. whereas the F'13*capR*$^+$/*capR9* heterogenote was non-mucoid, a *capR9*/*capR*$^+$ partial diploid was mucoid. By cloning the *capR*$^+$, *capR6* and *capR9* alleles from the F' plasmids on to a suitable multicopy vector (Zehnbauer & Markovitz, 1980), it has now been shown that *capR*$^+$ encodes a 94K protein with DNA-binding activity, whereas the *capR6* mutation leads to a deficiency in this protein. However, *capR9* encodes an altered protein of the same molecular weight which is produced in 5 to 10 fold amounts in excess of the *capR*$^+$ product. It has been further postulated that the *capR*$^+$ product is subject to autoregulatory control, a property which is lost with the *capR9* mutation.

In addition to the derepression of the enzymes associated with colanic acid synthesis, mutations in the *capR* locus have a number of pleitropic effects, e.g. UV sensitivity, low level resistance to tetracycline, chloramphenicol and puromycin. These properties are not the direct result of colanic acid synthesis *per se*, but reflect the diverse role of the *capR* gene product. *CapS* and *capT* mutants also have derepressed levels of the colanic acid biosynthetic enzymes, but do not share the latter properties associated with *capR*. Hence there are several targets on the *E. coli* chromosome whose function is to, directly or indirectly, switch on or off the genes involved in the colanic acid biosynthetic pathway.

P. aeruginosa alginate is an entirely different polymer from colanic acid, hence the control of the biosynthetic pathways may be different. Nevertheless, a similar genetic approach to the problem was likely to provide useful information.

Prior to the commencement of studies reported in this thesis we had identified two different phenotypic classes of mucoid strains, based on the production of mucoid growth on minimal agar (Vogel & Bonner, 1956). Strains belonging to group 1, e.g. PAO579 and PAO581, are clearly mucoid on both media after 24 h at 37°C, whereas group 2 strains, e.g. PAO568 and PAO578 appear rough and non-mucoid on minimal agar, but mucoid on complex media (Fyfe & Govan, 1980).

Early mapping studies, using FP2 and R68.45-mediated plate matings indicated that the mucoid characteristic (Muc), could be transferred between PAO strains by means of conjugation, but could not be directly selected for. FP2-mediated interrupted matings confirmed that one particular muc mutation, muc-36, was linked to the chromosomal markers his-5075 and cys- 5605 (at that time located at 12 mins on the PAO map), and was apparently distal to those markers with respect to the FP2 origin (Fyfe & Govan, 1980). Unexpectedly, muc-36 showed no linkage to markers in the 15-20 min region of the chromosome, hence no further mapping of this locus was possible. However, the relocation of his-5075 and cys-5605 from 12 min to the chromosomal region close to the major PF2 origin (Soldati et al, 1984), resolved many linkage anomalies observed by ourselves and other workers, and allowed further progress to be made in the mapping of muc loci. The results of this form part of this thesis.

b) Antibiotic hypersensitivity

In the course of studying the intrinsic resistance of P. aeruginosa strain PAO, various loci associated with this characteristic have been mapped on the chromosome. Mills & Holloway (1976) reported the isolation of an aeruginocin-tolerant PAO mutant (tolA) which was also hypersensitive to aminoglycoside antibiotics. This mutation mapped at approximately 10 mins on the chromosome map. A second locus associated with the resistance of P. aeruginosa to aminoglycosides (in particular kanamycin, neomycin, paromomycin and ribostamycin), has been designated aphA (Okii et al, 1983). This locus encodes an aminoglycoside inactivation enzyme and maps between 35 and 40 mins.

Intrinsic resistance of P. aeruginosa to the β-lactam antibiotics is based mainly on 2 mechanisms, hydrolysis by β-lactamases and exclusion of the antibiotics from their target sites in the inner membrane by the outer membrane (Richmond & Curtis, 1974 1974; Zimmerman, 1979). Several chromosomal loci associated with inducible β-lactamase production (bla) have been mapped (Matsumoto & Terawaki, 1982). Zimmerman (1979) has isolated a P. aeruginosa mutant (779/61) with increased sensitivity to a wide range of antibiotics including β-lactams, due to an apparent increase in outer membrane permeability. This mutant and its parent strain 799 have been utilized in various studies on the structural basis of the intrinsic resistance of P. aeruginosa (Zimmerman, 1980; Angus et al, 1982; Kropinski et al, 1982; Darveau & Hancock, 1983; Livermore, 1984). However, 799/61 was derived following multiple mutagenesis steps and attempts to determine the genetic basis of the antibiotic

hypersensitivity associated with this strain have so far been unsuccessful (Angus et al, 1982).

There have been several other reports of P. aeruginosa mutants with increased susceptibility to various antimicrobial agents (Noguchi et al, 1980; Kropinski et al, 1978; Ohmori et al, 1977), however, the interest in these strains was biochemical rather than genetic.

The genetic basis of antibiotic hypersensitivity as expressed in clinical P. aeruginosa isolates has not, to my knowledge, been reported. May & Ingold (1972) postulated that the intrinsic resistance of non-respiratory P. aeruginosa isolates might be plasmid-encoded, and that this plasmid may sometimes be lost during growth in the respiratory tract. However, no evidence was presented in support of this hypothesis.

c) Serum sensitivity and polyagglutinability

Alterations in LPS structure would appear to be the structural basis for these characteristics according to Hancock et al (1983) and Meadow et al (1984), thus mutations in certain genes associated with the biosynthesis of LPS would be expected to give rise to serum sensitivity and/or loss of the O-specific antigen. To my knowledge, there are no reported systematic genetic studies on LPS synthesis in P. aeruginosa, or the genetic basis of these characteristics in clinical isolates.

However, there are a number of reports on PA strains arising from strains of various serotypes following prolonged storage at

room temperature (Homma et al, 1972; Kawaharajo, 1973). In addition, it has been reported that lysogeny with certain phage could cause changes in the somatic antigens of P. aeruginosa (Liu, 1969; Bergen & Midtvedt, 1975).

CHAPTER V

EPIDEMIOLOGICAL TYPING OF *P. AERUGINOSA* STRAINS FROM CHRONIC RESPIRATORY INFECTIONS

There are several systems available for the epidemiological typing or "fingerprinting" of *P. aeruginosa* strains, including biotyping, antibiograms, serotyping, phage typing and bacteriocin (pyocin) typing (Brokopp & Farmer, 1979). In general, typing is performed to establish relationships between different *P. aeruginosa* isolates, usually associated with an outbreak of infection, in order to determine the origin and route of infection.

In the case of *P. aeruginosa* isolates from chronic respiratory tract infections, we are not primarily concerned with tracking down the source of strains, but rather in establishing the relationships between heterogeneous isolates from single and consecutive sputum specimens. If genetic studies on the characteristics associated with these isolates are to be meaningful, it is essential to demonstrate that isolates expressing different characteristics are closely related, having evolved *in vivo*, and not merely present in the same specimen due to infection with multiple, unrelated strains.

According to Brokopp & Farmer (1979), an ideal typing system must be based on a stable genetic property of the organism. However, apparent genetic stability is dependent on how well adapted the bacterial population is to its environment. If the population is in the process of adapting, due to strong selective pressures,

certain properties may be extremely unstable. Consequently, a system which is adequate for the typing of strains from one ecological niche may be totally inadequate for the typing of strains from another.

The purpose of this chapter is not to review the literature associated with the typing of P. aeruginosa, as this has been covered elsewhere (Bergan, 1975; Govan, 1978; Lanyi & Bergan, 1978; Bergan, 1978; Brokopp & Farmer, 1979; Pitt, 1980). Rather it is to examine the biological basis of the various available typing systems and to consider which would be the most useful for determining the relationships between strains expressing characteristics such as alginate synthesis, antibiotic hypersensitivity, serum sensitivity and PA, all of which potentially alter the cell surface.

Biotyping

Various definitions of biotyping have been used, ranging from characterization of the entire genotype and phenotype of the organism based on all the biological properties which can be measured, to the more restricted definition preferred by Brokopp & Farmer (1979) as the measurement of the following characteristics: colony type, haemolysis of red blood cells from different animals, pigment production, growth on selective media, production of a characteristic odour, and results of biochemical tests required for identification.

P. aeruginosa is considered to be a fairly "tight" phenotypic species, especially on the basis of physiological and nutritional characteristics (Stewart & Widanapatirana, 1981; Brokopp & Farmer,

1979). However, because there is considerable variation between strains on the basis of colonial morphology, pigment production etc., biotyping is sometimes useful.

Severe limitations with respect to the reliability of biotyping, used on its own for epidemiological purposes, have been reported by Bobo et al (1973). In this study, colonial dissociants of an epidemic P. aeruginosa strain gave extremely variable results on the basis of pigment production, antibiotic sensitivity and haemolysis of sheep red blood cells. Gaby (1946) and Zierdt & Schmidt (1964) have also described the different characteristics often associated with different colony types obtained from a single strain. On the basis of these observations, biotyping would not be appropriate for the typing of P. aeruginosa isolates from chronic respiratory tract infections, as different colonial types would be considered to be different strains.

Antibiograms

The antibiogram of a bacterial strain is defined as the susceptibility of that strain to a range of antimicrobial agents, usually determined by standardized single disk methods (Brokopp & Farmer, 1979). However, the differences in antibiotic susceptibilities encountered between different P. aeruginosa isolates from single sputum specimens (Seale et al, 1979; Thomassen et al, 1979) suggest that this would be an unsuitable typing method for determining the relationships between such isolates.

Serological typing

Various different schemes have been developed for the serological typing of P. aeruginosa (reviewed by Lanyi & Bergan, 1978), the majority of which are based on detection of the heat stable O-antigens. The scheme for O-serotyping used most extensively in this country is the International Antigenic Typing Scheme (IATS) which defines a total of 21 serotypes (Pitt, 1980). Construction of systems based on heat labile (H) antigens have also been attempted with varying success (Lányi & Bergan, 1978; Pitt, 1980), however all tend to give poor differentiation when used alone.

Lányi & Adam (1973) (cited by Lányi & Bergan, 1978) demonstrated that the P. aeruginosa O-antigens corresponded with phenol extracted LPS. Chester et al (1973) further showed that serological specificity is limited to the high molecular weight fraction, thought to correspond to the polysaccharide side chains, and Koval & Meadow (1975) reported that some amino sugars were specific for certain antigens.

O-serotyping is an extremely quick and reliable method which gives reasonable differentiation for the majority of P. aeruginosa clinical isolates. However, Penketh et al (1983b) have reported that only 32% of P. aeruginosa isolates from CF patients were typable using this method, due to loss of the specific O-type reaction and expression of PA. In a study on colonial dissociation in clinical P. aeruginosa isolates, Sheehan et al (1982) reported a sputum isolate which displayed serological non-identity with its colonial segregants, i.e. of the three colonial morphotypes

identified, two were serotype 0:11 and the other was untypable. No such dissociation was observed among blood isolates in this study. Thus it is apparent that serotyping is not the method of choice for establishing relationships between P. aeruginosa isolates from chronic respiratory tract infections.

Bacteriophage typing

Various phage typing procedures have been developed for P. aeruginosa (reviewed by Bergan, 1978), and most of these are based on the sensitivity of the bacterial strains to a set of virulent phages. The interaction between phage and bacterium is dependent on the presence of a specific receptor site on the bacterial surface.

One of the most extensively used sets of typing phages (a basic set of 18 phages) was first described by Lindberg & Latta (1974), and recently, the bacterial receptor sites for most of these phages have been partially characterized (Mutharia & Hancock, 1982). According to these workers, the receptor sites of phages 44, 109, 352, 1214, and F8 reside in the LPS, whereas others have protein or pilus receptors.

On the basis that serum sensitive and PA strains of P. aeruginosa probably have altered LPS, sensitivity to phages with LPS receptor sites may be altered. In addition, difficulties in the phage typing of mucoid strains have been reported (Bergan, 1978). Consequently, the use of phage typing for P. aeruginosa will not be pursued further in this thesis.

Pyocin typing

(i) On the basis of pyocin production. Pyocins (aeruginocins) are the bacteriocins of P. aeruginosa, and so far three main categories have been identified. R-pyocins are morphologically similar to the tails of contractile phages (Govan, 1974 a, b) and in some cases show immunological cross-reactivity with various phages (Ito & Kageyama, 1970). F-pyocins are flexuous structures which resemble tails of non-contractile P. aeruginosa phage (Govan, 1974b). The third category is made up of the low molecular weight (approximately 10^5 daltons) S-pyocins (Ito et al, 1970) which can be distinguished from the particulate varieties (i.e. R-pyocin and F-pyocin) on the basis of the size of the inhibition zone produced against a sensitive P. aeruginosa strain on agar medium. R and F-type pyocins are not readily diffusible and give rise to a narrow zone of inhibition, whereas S-pyocins produce a wide zone of inhibition. S-pyocins are generally considered to be trypsin sensitive, however a trypsin-resistant class has also been identified (Govan, 1978).

The most widely used and recommended method for typing, based on pyocin production, is that described by Govan & Gillies (1969) and Govan (1978). This method uses a cross-streaking technique for the detection of pyocin activity against 13 indicator strains, and gives fairly good strain discrimination with over 100 pyocin 'types' isolated, but has a number of technical disadvantages. These include (i) the 48h period required to obtain a result; (ii) the need to remove the producer strain growth before application of the

indicator strains, which is both messy and time-consuming; (iii) the inability to reliably type mucoid P. aeruginosa in the absence of a modification (Williams & Govan, 1973), which is also extremely time consuming and labour intensive, and impractical when large numbers of strains are to be typed. However, the advantage of pyocin typing from the point of view of establishing relationships between serum sensitive and PA strains is that it does not rely on the structure of cell surface components.

(ii) On the basis of pyocin sensitivity Several methods for typing P. aeruginosa on the basis of sensitivity to a set of pyocin preparations have been described (Tagg & Mushin, 1973; Rampling et al, 1975). However, like phage, the receptor sites for pyocins are cell surface components, and in the case of R-pyocins reside in the core part of the LPS (Meadow & Wells, 1978). The receptors of F- and S-pyocins are less well characterized.

In conclusion, it appears that typing based on pyocin production would provide the most meaningful results in the case of P. aeruginosa isolates from chronic respiratory infections. However, various technical aspects of the present published pyocin typing method (Govan, 1978) could be improved and it would be an advantage if the time required to obtain a result could be reduced.

Aims of this thesis

The research presented in this thesis forms part of an ongoing programme studying the epidemiological typing of *P. aeruginosa*, and the association of *P. aeruginosa* with chronic respiratory infection in CF patients.

The phenotypic heterogeneity of *P. aeruginosa* isolates from single and consecutive sputum specimens from CF patients, and the emergence of strains expressing unusual characteristics such as alginate synthesis and antibiotic hypersensitivity, suggest that adaptability is a significant factor for *P. aeruginosa* in this important and debilitating type of chronic infection. Hence, the major aim of this thesis was to examine the genetic basis of this adaptability. However, prior to the initiation of a genetic analysis, it was important to confirm that this observed heterogeneity was not merely due to mixed infection with multiple unrelated strains.

The areas of research covered in this thesis form three distinct sections, with the following specific aims:-

1. To modify the pyocin typing method previously developed inour laboratory (Govan, 1978), to overcome certain technical disadvantages, and to improve the discriminatory power of the present technique, by taking into account not only the presence or absence of pyocin activity, but also the nature of the pyocins (i.e. particulate or diffusible) produced. Results presented in Chapter VII.

2. a) To apply the classical genetic techniques of conjugation and transduction to the precise mapping of (*muc*) loci associated with the normal repression of alginate synthesis in *P. aeruginosa* strain PAO.

   b) To study the control of alginate synthesis associated with one particular *muc* locus, through the use of R' plasmids.

   c) To use an R'*muc*$^+$ plasmid to compare *muc* mutations in PAO and clinical mucoid strains. Results presented in Chapter VIII.

3. To determine the genetic basis of antibiotic hypersensivity in strain 492c and several other respiratory isolates, and to compare this form of hypersensitivity with the antibiotic hypersensitivity of the laboratory mutant 799/61 derived by Zimmerman. Results presented in Chapter IX.

MATERIALS AND METHODS

CHAPTER VI

A. MEDIA AND REAGENTS

1. Complex Media

Nutrient Broth (NB) was Oxoid No. 2 CM67.

Nutrient Yeast Broth (NYB) was Oxoid No. 2 supplemented with0.5% (w/v) yeast extract (Oxoid L21)

Nutrient Agar (NA) was Oxoid Columbia Agar Base CM331.

Pseudomonas Isolation Agar (PIA) was manufactured by Difco.

Diagnostic Sensitivity Test Agar (DSTA) was Oxoid CM261.

Tryptone Soya Agar (TSA) was Oxoid CM131.

Tryptone Soya Blood Agar (TSBA) was TSA supplemented with 5% defibrinated human blood.

Desoxycholate Citrate Agar (DCA) was Oxoid CM227.

MacConkey Agar (MCA) was Oxoid CM7b.

Semi-solid Agar Layers (SSA) were 1% Bactopeptone (Difco) in 0.5% Bacteriological agar (Oxoid L11).

All commercial media were prepared according to the manufacturers' instructions. Media were sterilized by autoclaving at 15 psi for 15 min.

2. Minimal Media

Minimal Agar (MA) as described by Vogel & Bonner (1956) was used in genetic experiments when auxotrophic markers were selected.

P medium (Leisinger et al, 1972), containing 20mM L-proline as the sole carbon and nitrogen source was used for testing strains carrying pru markers.

O medium (made according to P medium), containing 20mM L-ornithine as the sole carbon and nitrogen source was used for testing strains carrying oru markers.

3. Amino Acid Supplements

Amino acids were added to MA to give a final concentration of 1mM (except for isoleucine which was added to give a concentration of 50uM). Stock solutions of amino acids with a concentration of 50mM were prepared, sterilized by filtration and kept over chloroform.

4. Antibiotic Supplements

Antibiotics used were the following: ampicillin (Penbritin; Beecham), benzyl penicillin (Crystapen; Glaxo), carbenicillin (Pyopen; Beecham), cefoxitin (Mefoxin; MSD), cefuroxime (Zinacef; Glaxo), cephaloridine (Ceporin; Glaxo), chloramphenicol (Chloromycetin; Parke Davis), flucloxacillin (Floxapen; Beecham), gentamicin (Roussel), kanamycin (Bristol Laboratories), mecillinam

(Leo Pharmaceuticals), methicillin (Celbenin; Beecham), naladixic acid (Winthrop), novobiocin (Sigma), rifampicin (Roche), streptomycin (Glaxo), tetracycline (Glaxo), tobramycin (Lilley) and trimethoprim (Trimethoprim lactate*, Wellcome).

*1.3g of trimethoprim lactate is equivalent to 1g trimethoprim.

5. Preparation of Plate Media

Following sterilization by autoclaving at 15 psi for 15 mins, agar media were allowed to cool to 45-50°C before the addition of supplements, at the appropriate concentrations. All agar media, except TSA and TSBA were dispensed as 15ml volumes in plastic petri dishes. TSA plates (for use in pyocin typing by the spotting method) contained 10ml of medium in plastic petri dishes, whereas TSBA plates (for use in pyocin typing by the cross-streaking method) contained 20ml of medium in glass petri dishes. All plate media were dried at 48°C, inverted, without lids for approximately 15 min. Plate media were stored at 4°C. DSTA plates supplemented with antibiotics for use in MIC determinations were used with in 2 days.

6. Preparation of SSA

Following sterilization, the SSA was dispensed in 2.5ml volumes into Wasserman tubes and kept molten at 45°C until use on the same day.

7. Reagents

Physiological saline was 0.9% w/v NaCl.

Antisera for O-serotyping corresponded to the International Antigenic Typing Scheme (Lányi & Bergan, 1978) and were kindly donated by Dr Ty Pitt of the Division of Hospital Infection, Public Health Laboratory Service, Colindale.

Ethylmethanesulphonate (EMS) was manufactured by Koch-Light Laboratories Ltd.

B. BACTERIAL STRAINS, PLASMIDS AND PHAGE

The bacterial strains and plasmids used in each section of the research work are described at the beginning of the relevant Chapter in the Results. Phage F116L (Krishnapillai, 1971) was used for transductions, and phage F116c (Watson & Holloway, 1976), a clear-plaque mutant of F116L, was used for strain construction.

C. METHODS

1. Storage and propagation of bacterial strains

Longterm storage of bacterial strains was at -70°C in 10% w/v skim milk (Oxoid L31). Bacterial suspensions for storage in this way were prepared by emulsifying several single colonies from an NA or PIA plate in 1ml volumes of skim milk contained in plastic screw-top vials. Stock NYB bacterial cultures were used forinoculating NA, PIA or other selective media to obtain fresh single colonies for

experimental purposes. These stock cultures were obtained by inoculating a single fresh bacterial colony into a 1oz vial (McCartney bottle) containing 10ml NYB and incubating overnight at 37°C in an orbital incubator (Gallenkamp) rotating at 140 revs min^{-1}. Stock cultures were stored at 4°C and used within two weeks.

All NB and NYB bacterial cultures were incubated as above unless otherwise stated. Plate cultures were incubated at 37°C unless otherwise stated.

2. Storage and propagation of transducing phage

Longterm storage of phage preparations (in NB) was at -70°C. Stock preparations were maintained at 4°C. High titre preparations for transductions were obtained using the following procedure (plate harvest method): 0.1ml aliquots of a stock phage preparation (containing approximately 10^5pfu ml^{-1}) were added to 3 x 2.5ml volumes of molten SSA, previously seeded with 0.1ml aliquots of a NYB culture of the donor bacterial strain. The molten SSA was then overlayed on to 3 NA plates and allowed to set. Following 18h at 37°C, the plates showed just confluent lysis of the bacterial lawn and the layers were scraped into 10ml NB in a McCartney bottle. After standing at room temperature for 15 min, the mixture was vortexed on a rotamixer (Hook & Tucker Ltd.) and centrifuged at 5,000rpm for 30 min in a Bactifuge (Heraeus Christ). The supernatant was then filtered through a millipore filter (pore size 0.2μm) and assayed using the spot method. Transducing preparations were used within 2 weeks.

3. Pyocin typing by the cross-streaking method

The procedure used was described by Govan (1978).

As suggested in this review, the recognition of S-pyocin activity was incorporated into the typing scheme. Thus, a test strain was allocated to a pyocin type not only on the basis of the pattern of inhibition observed against the 13 standard indicator strains, but was further characterized by noting the presence of classic S-pyocin activity which causes a zone of inhibition which extends beyond the original growth area of the producer strain. Therefore, a strain of, for example, pyocin type 1/a producing S-pyocin against indicator strains 7 and B, is designated type 1/a ($S_{7,B}$).

4. Pyocin typing by the spotting method

Strains of P. aeruginosa to be typed were streaked for single colonies on to NA and incubated at 37°C overnight. The single colonies arising from each test strain were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1ml of sterile physiological saline.

A multipoint inoculator (Model A400: Denley Instruments Ltd., Sussex, England) was used to dispense 1µl volumes of the bacterial suspensions onto a set of 13 plates (diameter 90mm), each containing 10ml of TSA. In this way, 20 strains could be typed simultaneously against each indicator strain. After the spots dried, usually within a few mintues, the inoculated plates were incubated at 30°C

for 6h. Subsequently, filter paper disks (50mm diameter: Whatman Inc., England) were impregnated with chloroform, and the plates were placed over the disks (ensuring that the disks did not come into contact with the plates) for 15 min to allow the chloroform vapour to kill the bacteria. The plates were then exposed to air for 15 min to eliminate residual chloroform vapour. Cultures of the indicator strains, grown without aeration in NB for 4h at 37°C (giving a cell density of approximately 10^7 organisms ml^{-1}) were applied to the plates by adding 0.1ml of each indicator culture to 2.5ml of molten SSA held at 45°C and poured as overlays (i.e. a separate indicator strain was applied to each of the 13 plates). When the overlays had set (within 1 min), the plates were incubated for 18h at 37°C. Pyocin types were determined as described for the cross-streaking method.

5. Induction of pyocin production by exposure to UV

The method used was based on that described by Kageyama (1975). Plates inoculated with the producer strains (as described for the Spotting method of pyocin typing) were incubated at 30°C for 4h. Plates were then exposed to UV irradiation using a Phillips TUV 30 watt bulb (output 20µ watts cm^{-2}) for 15 secs. Following a further 2h incubation at 30°C, pyocin production was detected and described for the Spotting method.

6. The killing of producer strains by exposure to UV

Producer strain growth resulting on the TSA plates after 6h at 30°C was exposed to UV irradiation (20µ watts cm^{-2}) for 15 mins.

7. Serotyping

This was performed using the slide agglutination method as described by Lányi & Bergan (1978), except that the test strains were grown on NA rather than blood agar.

8. The spot method for the assay of phage

A dry NA plate was flooded with a 10-fold dilution of an 18h NYB culture of the propagating bacterial strain. After removal of excess culture, the plate was left to dry at room temperature. Serial 10-fold dilutions of the phage preparation were made in NB (from 10^1 to 10^9). 20µl volumes of the 10^6, 10^7, 10^8 and 10^9 dilutions were applied to the surface of the seeded plate using a micropipette (Finnpipette, Jenkins Scientific Ltd). After the drops had dried the plate was incubated for 18h at 37°C and the phage titre estimated by counting the plaques from a dilution giving rise to 20-200 such plaques.

9. Isolation of auxotrophic mutants

Auxotrophic mutants were obtained following EMS mutagenesis (Waton & Holloway, 1976) and carbenicillin enrichment (Watson & Holloway, 1978).

10. Isolation of mucoid mutants

Spontaneous mucoid mutants were obtained using the carbenicillin semi-selection method described by Govan & Fyfe (1978) or by the phage selection method (Govan, 1975) using phage F116c.

11. Isolation of non-mucoid revertants

Spontaneous non-mucoid revertants were isolated according to Govan et al (1979).

12. Isolation of rifampicin resistant (RIF^r) mutants

Spontaneous RIF^r mutants were isolated by spreading 0.2ml aliquots of the appropriate 18h NYB bacterial culture onto three DSTA plates supplemented with 250µg rifampicin ml^{-1}, and incubating at 37°C for 24-48h.

13. Isolation of carbenicillin hypersensitive mutants

An exponential phase NYB culture was mutagenized with EMS (Watson & Holloway, 1976). The mutagenized culture thus obtained was diluted 100-fold in fresh NYB and incubated for 18h at 37°C (with agitation) to allow expression of mutations. This 18h culture was diluted 10^6 in saline and 0.1µl aliquots spread over a series of NA plates. The plates were allowed to dry and incubated for 18h at 37°C. The single potential mutant colonies thus obtained (50-200 per plate) were screened for hypersensitivity to carbenicillin by replica plating to DSTA plates containing 5µg carbenicillin ml^{-1} and

then to fresh NA plates (to provide master plates). Master plates and carbenicillin plates were incubated for 18h at 37°C and the colonies arising on both sets were compared. Potential carbenicillin hypersensitive clones failed to grow on DSTA + 5µg carbenicillin ml^{-1} but were present on the master plates. These were purified on NA and further characterized by determining the MIC of carbenicillin and other antibiotics.

14. Construction of strains carrying R68.45

Donor strains carrying the plasmid R68.45 were constructed and tested for donorability as described by Haas & Holloway (1976).

15. Plate matings between PAO strains

The procedure used for plate matings mediated by FP2 and R68.45 was described by Stanisich & Holloway (1972).

16. Plate matings between a non-PAO donor and a PAO recipient

The procedure used was essentially the same as for plate matings between PAO strains, except that NYB cultures of the PAO recipient strains were incubated for 18h at 43°C rather than 37°C, as growth at this temperature (i.e. 43°C) renders P. aeruginosa phenotypically restriction deficient (Rolfe & Holloway, 1966).

17. Transductions

The procedure used was described by Krishnapillai (1971).

18. Construction of strains using transductions

Transductions using F116c were performed as described by Krishnapillai (1971), however, transductants were purified several times on the selective medium and then on NA to remove residual virulent phage. Only transductants which remained sensitive to F116c were retained.

19. Characterization of recombinants or transductants.

The segregation of unselected markers was determined by partially purifying the recombinants (or transductants) by spotting the colonies to plates of the same medium on which the cross was performed (50 colonies/plate). After overnight growth at 37°C, these plates were used as master plates in replica plating or spotting to the appropriate medium for scoring the marker of interest, i.e. supplemented MA or MA for auxotrophic markers, P medium for Pru, O medium for Oru, PIA for Muc, DSTA supplemented with appropriate antibiotics for markers associated with antibiotic hypersensitivity, or resistance markers associated with R68.45 ($Muc^{+/-}$ could also be scored on DSTA).

20. Testing recombinants for sensitivity to UV

The construction of strains carrying the rec-102 marker required the screening of recombinants for coinheritance of one of the characteristics associated with rec-102, i.e. UV sensitivity (UV^s). Recombinants obtained in a plate mating between a rec-102 donor strain and a rec^+ recipient were partially purified as

described in the previous section and then patched onto duplicate NA plates. One of the NA plates was then exposed to UV irradiation (20μ watts cm^{-2}) for 5 seconds. Following 18h incubation of the plates at 37°C, UV^s recombinants on the irradiated plate gave rise to growth of single colonies only within the area of patch inoculation, whereas wild type recombinants gave rise to heavy confluent growth. UV^s recombinants were then purified from the non-irradiated NA plate.

21. Patch matings

Patch matings were used in the isolation of R' derivatives of R68.45 and were performed as described by Holloway (1978).

22. Broth matings

The transfer of R68.45 and R' plasmids between bacterial strains was performed using broth matings. 1ml of a 5h NYB culture of the donor strain was added to 1ml of an 18h NYB culture of the recipient, and the mating mixture incubated for $1\frac{1}{2}$h at 37°C without agitation. The mating mixture was then either streaked for single colonies on to appropriate selective plate medium, or serially diluted in physiological saline and 0.1ml of the appropriate dilutions spread on to selective medium. Selective plates were incubated at 37°C for 48h.

23. Antibiotic sensitivity testing

Exponential phase NYB cultures of the bacterial strains were diluted in physiological saline to a concentration of approximately 10^5 organisms ml^{-1}. A multipoint inoculator (Mode A400: Denley Instruments Ltd.) was used to dispense aliquots (containing 10^2-10^3 cells) on to DSTA plates appropriately supplemented with the antibiotics. A control DSTA plate containing no antibiotic was also included. The MIC was read as the lowest concentrationof antibiotic causing complete inhibition of growth after 18h incubation at 37°C.

24. Testing bacterial strains for serum sensitivity

The procedure used was described by Penketh et al (1983a).

RESULTS

CHAPTER VII

DEVELOPMENT OF A REVISED PYOCIN TYPING METHOD FOR P. AERUGINOSA

Bacterial Strains. Fifty pyocinogenic strains of P. aeruginosa from the laboratory culture collection were employed as producers in the development of the revised (spotting) pyocin typing method, and these strains were used to compare the results obtained using this method with those obtained with the standard cross-streaking method. Thereafter, an additional 500 clinical isolates of P. aeruginosa, from the Royal Infirmary of Edinburgh and the Royal Hospital for Sick Children, Edinburgh, including mucoid isolates from CF patients and PA strains, were used to evaluate the spotting method. The 13 indicator strains 1 to 8 and A to E were those described by Govan & Gillies (1969).

1. Development of the Spotting Method for Pyocin Typing

The spotting method as described in Materials & Methods is shown in diagrammatic form in Figure 3. The essential aspects of the method can be summarized by the following:- (i) Producer strain suspensions, containing 10^8 to 10^9 bacteria ml^{-1}, are applied to standard plates containing 10ml TSA, using a multipoint inoculator. (ii) Following inoculation, the plates are incubated for 6h at 30°C to allow producer strain growth. (iii) Producer strain growth is killed using chloroform vapour. (iv) NB cultures of the indicator strains, previously incubated at 37°C for 4h, are incorporated into

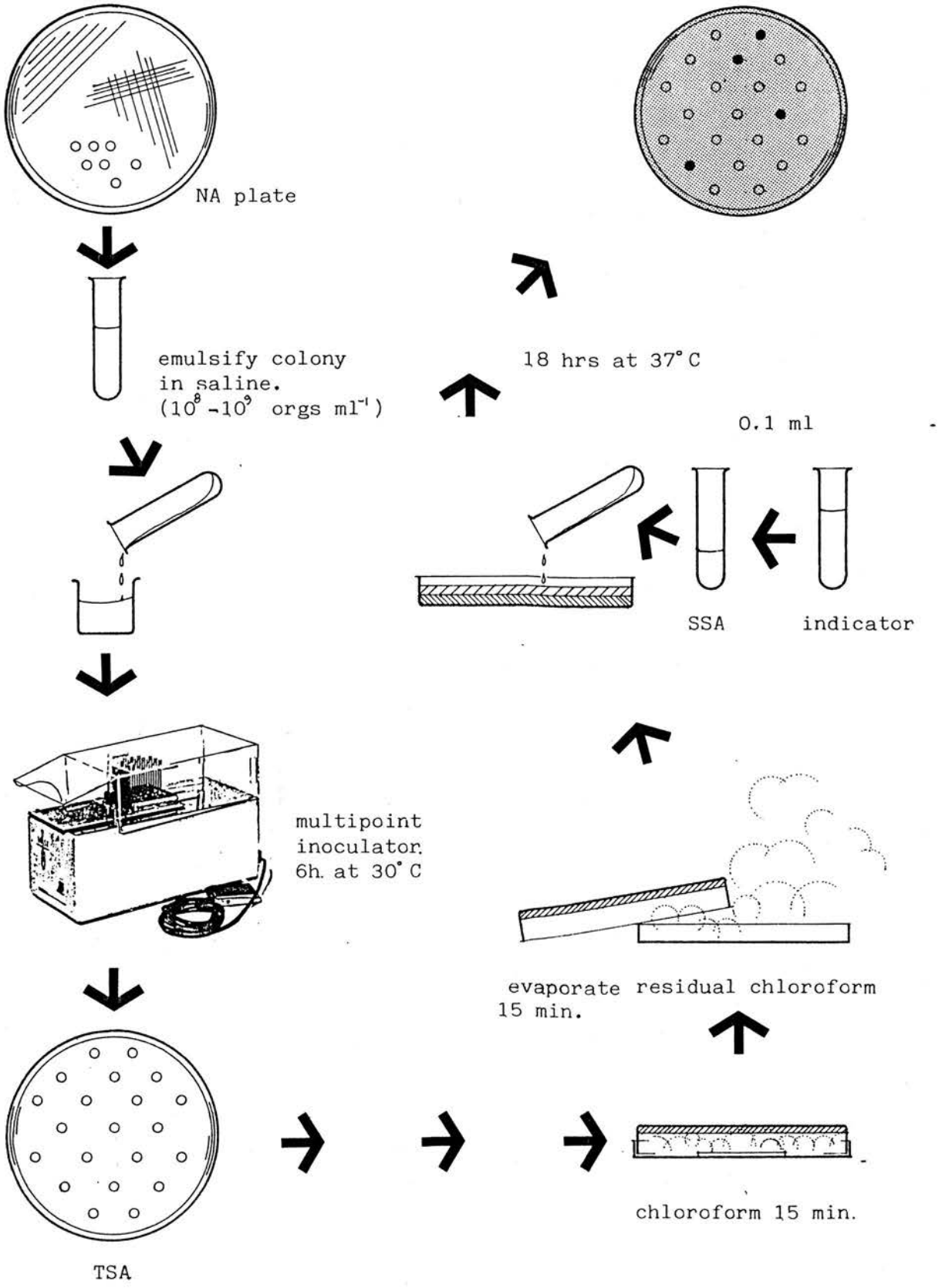


Figure 3 The spotting method of pyocin typing.

SSA overlays, and applied to the typing plates. (v) The typing plates are incubated for 18h at 37°C.

Certain aspects of the spotting method are based on the cross-streaking method as described by Govan (1978), e.g. the use of TSA for producer strain growth, incubation of producer strains at 30°C, the use of chloroform vapour to kill the producer strains, and the cultural conditions used for the indicator strains. The idea for using a spot inoculation technique and a 6h incubation time for the producer strains was based on the method for pyocin detection described by Kageyama (1975).

Figure 4 illustrates the four main inhibition zones detectable using the spotting method. The zone of bacterial growth following inoculation with the multipoint inoculator and 6h incubation was generally 5mm diameter. If the test strain failed to produce detectable pyocin against an indicator, this area of growth was visible beneath the indicator lawn, but there was no inhibition of the indicator. Production of R- or F-pyocin by the test strain resulted in an inhibition zone 5 to 7mm in diameter, with a sharp edge. S-pyocins generally produced inhibition zones of 9 to 12mm diameter, often with a diffuse edge. In addition to pyocin activity, phage activity could often be detected using the spotting method, characteristically giving rise to a mottled or "moth-eaten" inhibition zone. The recognition of phage activity was often useful as an additional epidemiological marker.

Figure 5 shows two typical routine typing plates obtained using the spotting method.

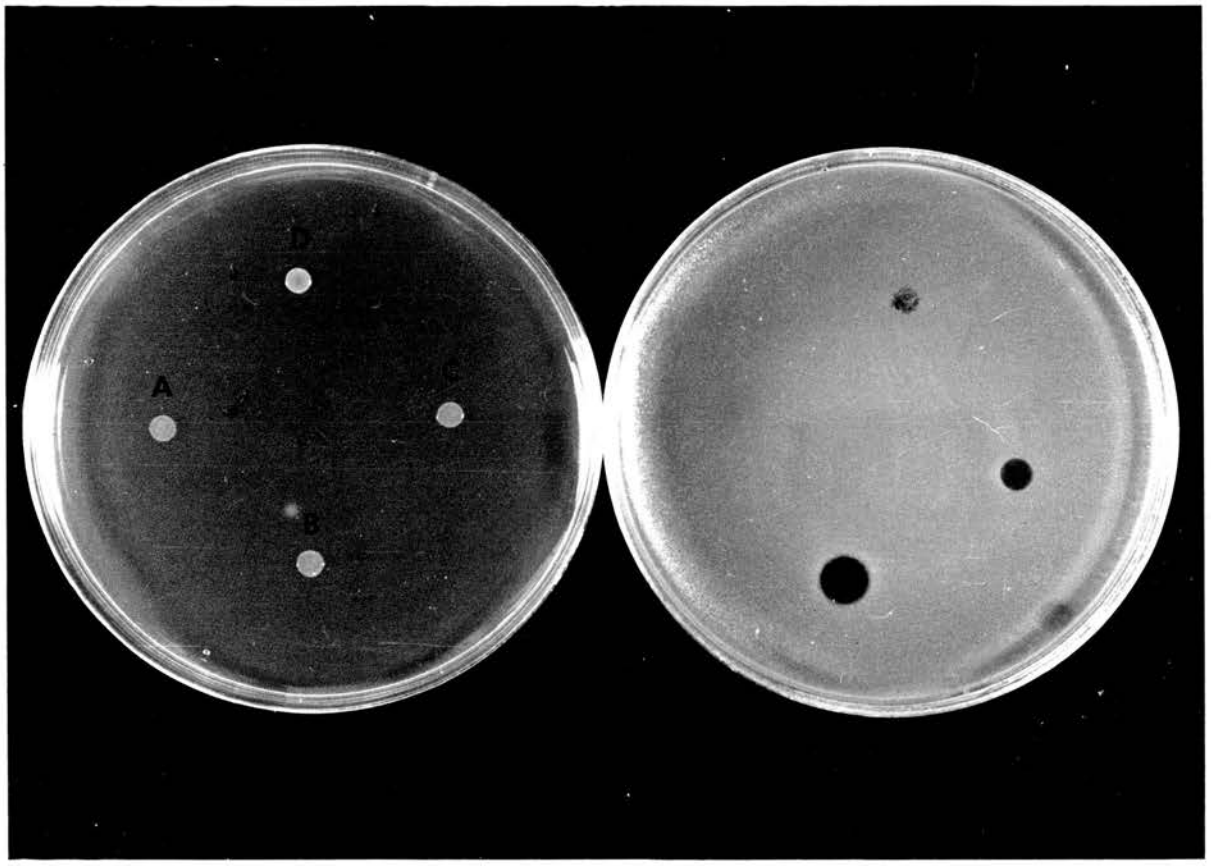


Figure 4 Examination for pyocin activity against indicator strain 8 in four strains of P. aeruginosa by the spotting method. The left plate shows the test strains A, B, C and D after 6h at 30°C and before addition of the indicator strain. The right plate shows the inhibition zones associated with the same four strains after addition of the indicator strain in an agar overlay, and subsequent incubation for 18h at 37°C. Strain A shows no pyocin activity(-); strain B shows S-pyocin activity (+s) characterized by an inhibition zone extending beyond the area of original growth; strain C shows a restricted inhibition zone characteristic of R- and F-pyocins (+); strain D shows a mottled inhibition zone typical of phage activity (-ϕ).

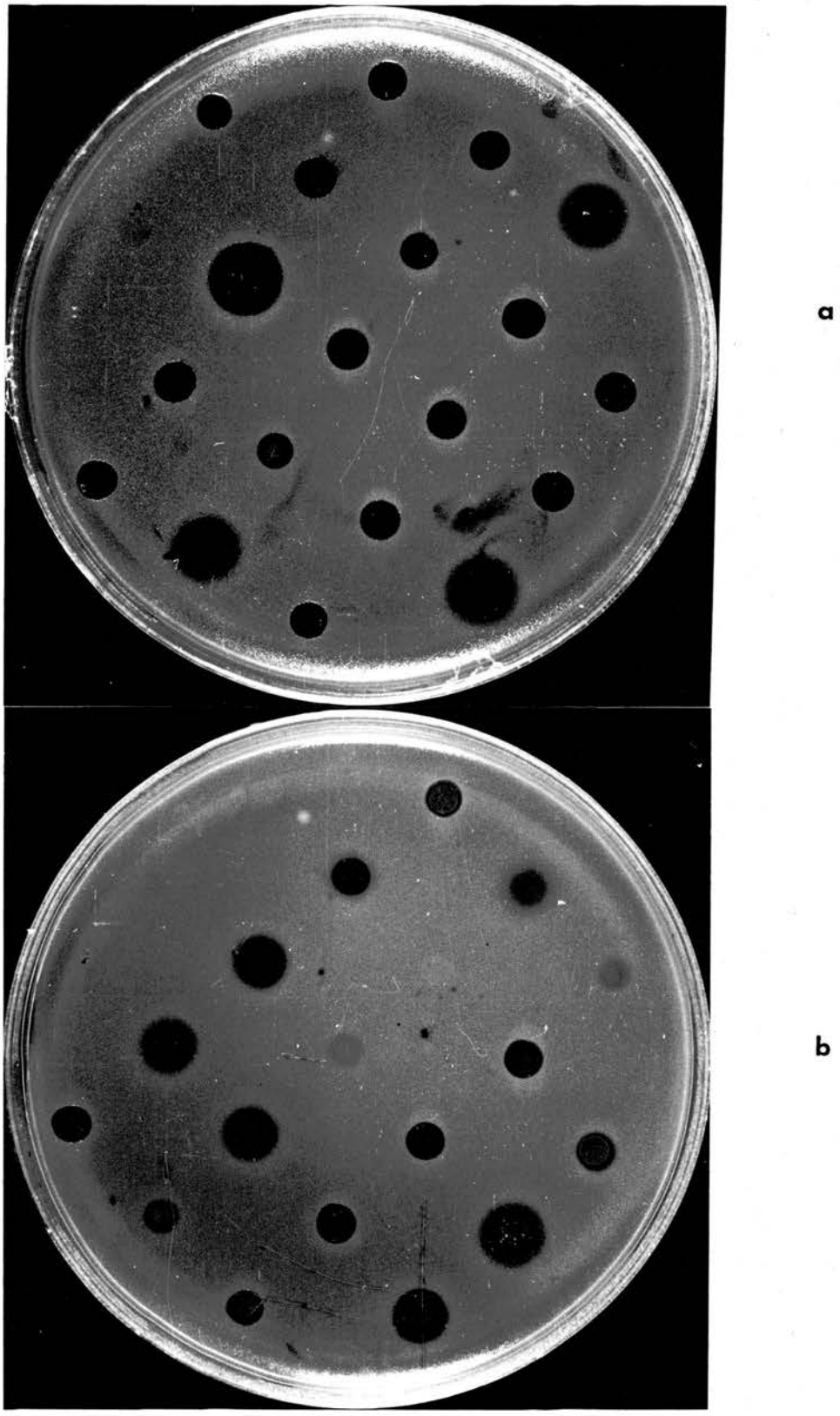


Figure 5 Typical routine typing plates obtained by the spotting method. (a) Indicator strain 5; (b) indicator strain B.

During the development of the spotting method, certain variables were assessed for their effect on pyocin production and detection. These results are summarized in Table 2.

2. Evaluation of the Spotting Method

2a. Comparison between spotting and cross-streaking methods

For comparative purposes, 50 strains of P. aeruginosa were pyocin typed using both methods. Of these 50 strains, 27 gave identical results using the two methods, including the detection of S-pyocin activity. Nine strains gave the same pyocin type, but the cross-streaking method failed to detect S-pyocin activity against one or more of the indicators. Fourteen strains gave a different pyocin type depending on the method used; in 12 strains, the spotting method detected pyocin activity against certain indicators which the cross-streaking method failed to detect, and in the remaining 2 strains, pyocin activity was detected by the cross-streaking but not the spotting method.

2b. S-pyocin production as a further epidemiological discriminator

An additional 500 clinical isolates of P. aeruginosa were pyocin typed by the spotting method. Ninety-nine percent of these strains were typable and 74% produced S-pyocin activity against one or more of the 13 indicator strains. All indicator strains showed some degree of sensitivity to S-pyocin activity and amongst the 500 strains typed, 63 different "S-patterns" were distinguished,

TABLE 2 The effects of varying particular aspects of the spotting method on pyocin production and detection.

Variation in method	Result
Substitution of NA for TSA	No consistent differences
Use of a larger test strain inoculum (i.e. suspension approx. 10^6 cells ml^{-1}	Too much growth after 6h resulting in an uneven overlay lawn
Incubation of test strains for 18h	Needed to remove test strain growth prior to pouring indicator overlays. In some cases, S-pyocin activity was not detectable
Incubation of test strains for 37°C	Variable. Most pyocins produced at both temperatures. Certain S-pyocins apparently not produced at 37°C (or may have been degraded by other bacterial products)
Induction of pyocin production in test strains by UV	No advantage. Some pyocins inhibited. Phage induction.
Killing test strains with UV	Poor result. Phage induction.

suggesting that there are many different S-pyocins and/or many clinical P. aeruginosa isolates produce multiple S-pyocins. The most common "S-patterns" were S_5 (11.3% of those strains exhibiting S-pyocin activity), $S_{5,B}$ (9.7%), S_7 (8.9%) and $S_{7,B}$ (8.6%).

As an example of the improved discrimination provided by the recognition of S-pyocin activity, 37 clinical P. aeruginosa isolates belonging to the common pyocin type 3/e could be subdivided into 6 groups on the basis of their S-pattern (see Table 3). It was also interesting to note that serotyping was of no value for the subdivision of these 3/e strains as 33 of the 37 were serotype 0:6 and the remaining 4 were PA.

2c. Typing of mucoid P. aeruginosa by the spotting method

Thirty mucoid isolates of P. aeruginosa from the sputa of six CF patients were pyocin typed by the spotting method. These included multiple isolates from single sputum samples. All 30 strains gave clear typing patterns with 13 strains producing S-pyocins against one or more of the indicators. Multiple isolates (differing on the basis of antimicrobial susceptibilities) from five of the patients were shown to be of the same pyocin type (different types for each patient), whereas the sixth patient harboured strains of 2 distinct types, 29/f (S_5) and 13/k.

2d. Typing of PA strains

During the course of these studies a number of the P. aeruginosa strains were shown to be $^{PA}_{\wedge}$ and hence not able to be

TABLE 3 The subdivision of 37 *P. aeruginosa* isolates of pyocin type 3/e on the basis of S-pyocin production.

S-pattern	No. of strains
No S activity	11
S_5	8
S_2	6
S_E	6
S_B	4
$S_{2,5}$	2
Total	37

assigned a particular serotype. In each case, pyocin typing provided a means for the reliable epidemiological fingerprinting of these strains.

CHAPTER VIII

FURTHER STUDIES ON THE GENETIC BASIS OF ALGINATE SYNTHESIS IN P. AERUGINOSA

Bacterial Strains and Plasmids. The P. aeruginosa strains and plasmids relevent to this chapter are described in Table 4.

1a) Further phenotypic characterization of mucoid P. aeruginosa

The early recognition of heterogeneity amongst mucoid mutants of P. aeruginosa strain PAO was based on differential expression of alginate synthesis by different strains on MA (Fyfe & Govan, 1980). Examples of the two phenotypic groups identified on this basis are illustrated in Figure 7. The addition of NA and DCA for the purposes of phenotypic characterization, increased the number of distinguishable "medium-dependent" mucoid groups from two to four (see Table 5). Similar phenotypic characterization of mucoid P. aeruginosa isolates from the sputa of 17 CF patients (100 colonies tested from each sputum sample), indicated that all these patients harboured strains belonging to group 1, 11 patients harboured group 2 strains, 6 patients yielded group 3 strains and 5 patients yielded group 4 mucoid strains in addition to their group 1 strain. Thus, all four groups appear to be clinically relevent, though on the basis of isolation frequency, groups 1 and 2 are probably of greater significance than groups 3 and 4.

TABLE 4 Strains and plasmids of *P. aeruginosa* relevent to this chapter

Strain	Genotype/Phenotype	Origin, Method of Construction, or Reference
PAO8	*met-28*, *ilv-202*, *str-1*	Isaac & Holloway, 1968
PAO38	*leu-38*	Stanisich & Holloway, 1961
PAO568	*leu-38*, *str-2*, *muc-2*, $FP2^+$	Fyfe & Govan, 1980
$PAO5_{68}RI$	*leu-38*, *str-2*, *muc-2* *Rev* $FP2^+$	"Suppressed" non-mucoid revertant of PAO568. See Section 1e.
PAO578	*leu-38*, *str-2*, *muc-22* $PF2^+$	Fyfe & Govan, 1980
$PAO578_{R11}$	*leu-38*, *str-2*, *muc-2* *Rev* $FP2^+$	"Suppressed" non-mucoid revertant of PAO578. See Section 1c.
PAO579	*leu-38*, *str-2*, *muc-23* $FP2^+$	Fyfe & Govan, 1980
PAO581	*leu-38*, *str-2*, *muc-25* $FP2^+$	Fyfe & Govan, 1980
PAO954	*met-9011*, *ami E200*, *oru-292*	Obtained from D. Haas
PAO961	*oru-319*	Obtained from D. Haas
PAO961 RIF^r	*oru-319* RIF^r	RIF^r mutant of PAO961
PAO964	*ami-151*, *hutC107*, *pru-354*	Meile *et al*, 1982
PAO1042	*pur-67*, *thr-9001*, *cys-59*, *proB65*	Royle *et al*, 1981
PAO1042 RIF^r	*pur-67*, *thr-9001*, *cys-59*, *proB65*, RIF^r	RIF^r mutant of PAO1042
PAO6502	*ami-151*, *hutC107*, *pru-354*, *muc-37*	Mucoid mutant of PAO964
PAO6502	*ami-151*, *hutC107*, *pru-354*, *muc-37*, RIF^r	RIF^r mutant of PAO6502

TABLE 4 (continued)

Strain	Genotype/Phenotype	Origin, Method of Construction, or Reference
PAO6503	ami-151, hutC107, pru-354, muc-38	Mucoid mutant of PAO964
PAO6504	ami-151, hutC107, pru-354, muc-39	Mucoid mutant of PAO964
PAO6505	ami-151, hutC107 pru-354, muc-40	Mucoid mutant of PAO964
PAO6506	pur-67, thr-9001, cys-59	Pro^+ transductant of PAO1042 obtained using F116c
PAO6513	leu-38, muc-45	Mucoid mutant of PAO38
PAO6514	leu-38, muc-52	Mucoid mutant of PAO38
PAO6519	pru-354, muc-37, rec-102	Described in Section 2a
PAO6527	thr-9001, muc-2	Cys^+Pur^+ muc-2 recombinant obtained in plate mating between PAO568 and PAO6506
PAO6529	thr-9001, muc-22	Cys^+Pur^+ muc-22 recombinant obtained in plate mating between PAO578 and PAO6506
PTO.66	his-4, lys-12, ilv-1118, trp-6, pro-82, rec-102	Royle & Holloway, 1981
492a RIF^r	Prototrophic, mucoid, RIF^r	RIF^r mutant of a clinical mucoid isolate
1949 RIF^r	Prototrophic, mucoid, RIF^r	RIF^r mutant of a clinical mucoid isolate
2246 RIF^r	Prototrophic, mucoid, RIF^r	RIF^r mutant of a clinical mucoid isolate
2263 RIF^r	Prototrophic, mucoid, RIF^r	RIF^r mutantof a clinical mucoid isolate
2249 RIF^r	"	"

TABLE 4 (continued)

Plasmid	Phenotype	Reference
R68.45	$CB^rTC^rKM^rTra$ Cma IncP-1	Haas & Holloway, 1976
pJF4	$CB^rTC^rPru^+Oru^+Muc^+$ Tra IncP-1	R' derived from R68.45. See Section 2b
pJF6	$CB^rTC^rKM^rPru^+$ Tra IncP-1	R' derived fromR68.45 See Section 2b
pJF54	$CB^rTC^rKM^rPru^+$muc-22 Tra IncP-1	R' derived from R68.45 See Section 2g
pJF55	$CB^rTC^rKM^rPru^+$muc-2 Tra IncP-1	R' derived from R68.45 See Section 2g
PJF56	$CB^rTC^rKM^rPru^+$muc-45 Tra IncP-1	R' derived from R68.45 See Section 2g

The genotype symbols used are the same as in Fig. 2. RIF^r designates resistance to rifampicin; CB^r, resistance to carbenicillin; Tc^r, resistance to tetracycline; KM^r, resistance to kanamycin; Tra, transferability; Cma, chromosome mobilizing ability.

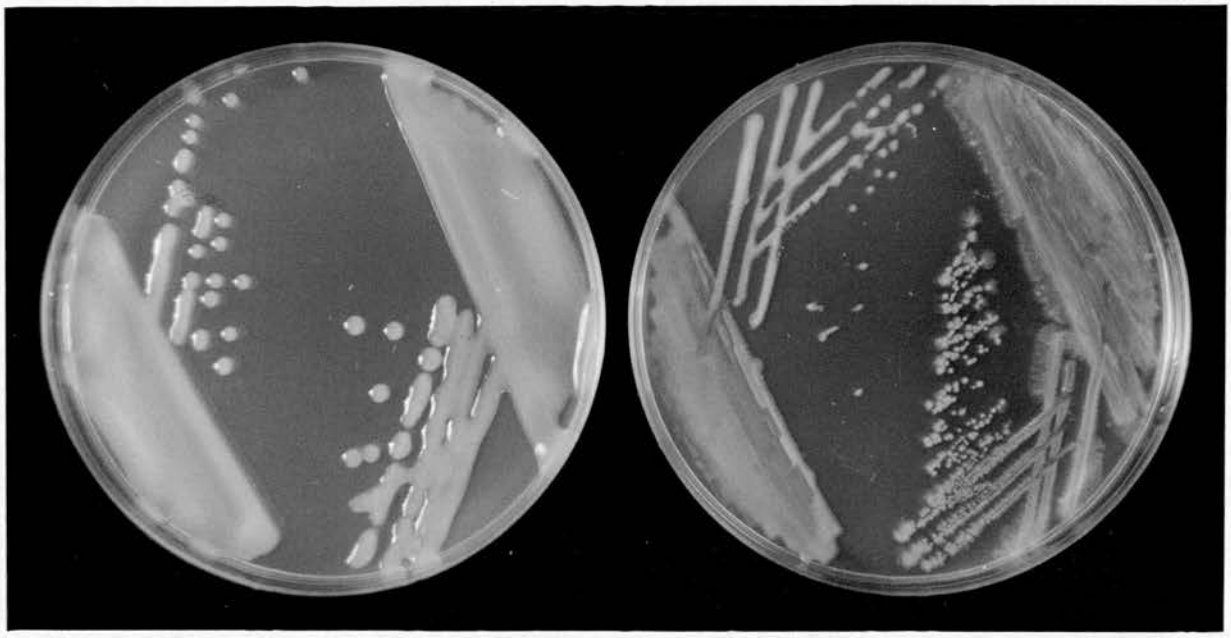


Figure 7 Two mucoid strains PAO568 and PAO579 after 24h incubation at 37°C on PIA (left plate) and MA supplemented with leucine (right plate). PAO579 (on the left of each plate) produces alginate on both media; whereas PAO568 (on the right of each plate) produces alginate on PIA, but apparently not on MA.

TABLE 5 Phenotypic characterization of mucoid strains of *P. aeruginosa* isolated *in vitro* and from patients with CF

Mucoid Group	Type of growth on indicated medium*			
	PIA	NA	DCA	MA
1	+	+	+	+
2	+	+	+	-
3	+	+	-	-
4	+	-	-	-

*
+ = mucoid growth resembling Phillips colonial type 5 (Phillips, 1969) after incubation at 37°C for 24h.

- = non-mucoid growth.

During the course of the genetic mapping studies (to be described), it became apparent that the number of mucoid phenotypic groups further increased with the addition of more growth media. For example, when five group 2 mucoid PAO strains were grown on MCA and P medium, four subgroups were identified. Table 6 summarizes the "medium-dependent" expression of alginate synthesis on MA, MCA and P medium associated with all muc mutations mapped in this study (all were associated with mucoid growth on PIA, NA and DCA). The phenotypic characteristics associated with a particular muc mutation was constant regardless of the genetic background of the strain carrying the mutation.

1b) The mapping of muc mutations by FP2-mediated and R68.45-mediated conjugation

Figure 6 shows the locations of genetic markers relevant to this study on the PAO map.

Previous studies had shown that muc-2, muc-22, muc-23 and muc-25 were all linked to cys-5605 and his-5075 by FP2-mediated conjugation (Fyfe & Govan, 1980), but the anomolous locations of these auxotrophic markers at the time, prevented precise mapping of the muc loci.

The relocation of cys and his, and the isolation of the recipient strain PAO1042 (Royle et al, 1981) with 3 auxotrophic markers (pur-67, cys-59 and proB65) in the vicinity of the major FP2 origin, suggested that matings between mucoid donors and PAO1042 might provide conclusive linkage data. Consequently, plate matings

TABLE 6 The "medium-dependent" expression of alginate synthesis associated with different *muc* mutations

	Type of growth on indicated medium*		
muc mutation	MA	MCA	P
muc-2	-	+	+
muc-22	-	+	+
muc-23	+	+	+
muc-25	+	+	+
muc-37	-	-	+
muc-38	-	+	-
muc-39	-	-	+
muc-40	-	+	-
muc-45	+	+	+
muc-52	+	+	+

* + = mucoid growth; - = non-mucoid growth

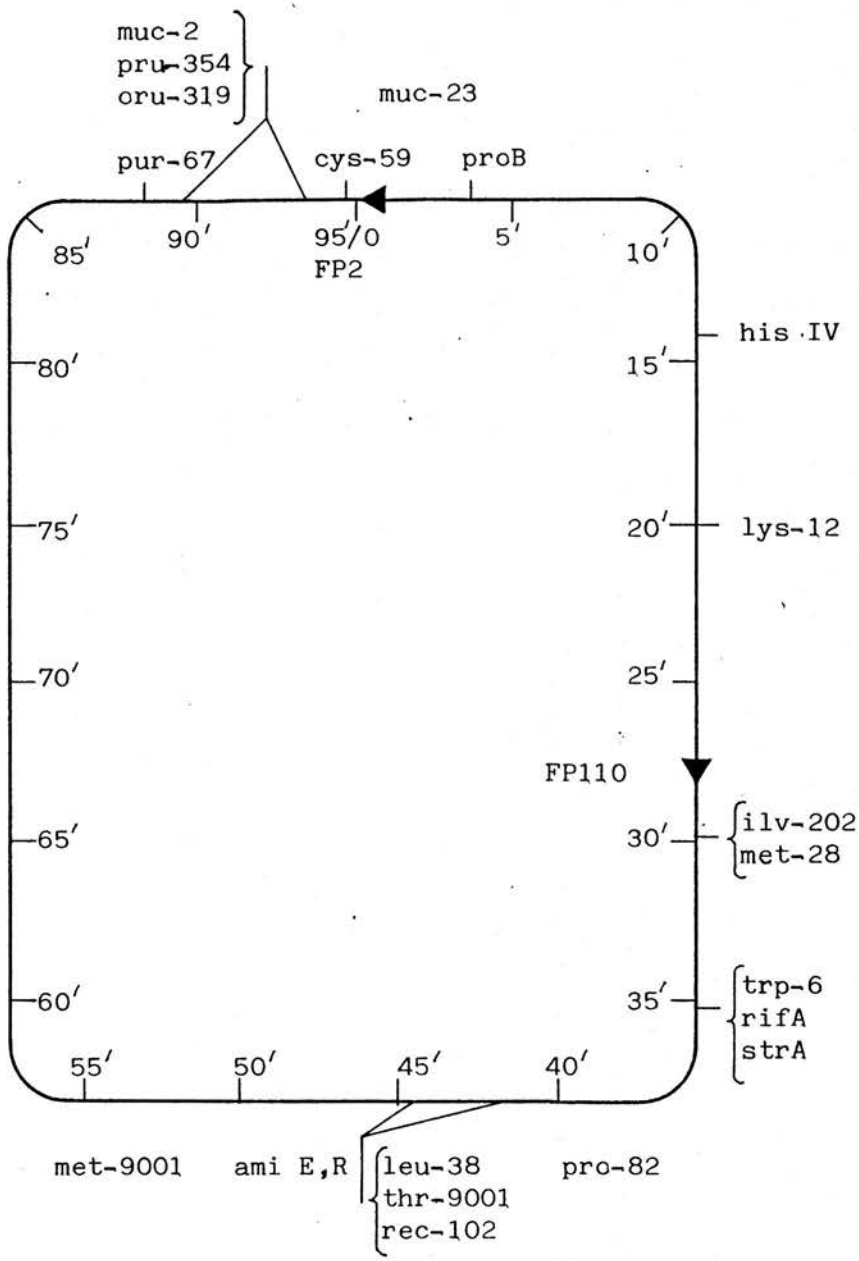


Figure 6 The P. aeruginosa PAO chromosome map showing the locations of markers relevent to this chapter. Marker designations are the same as for Fig. 2.

were performed between PAO1042 and the mucoid strains PAO578 (muc-22) and PAO579 (muc-23), selecting for Pur^+, Cys^+ and Pro^+ recombinants. The recombinants obtained for each selected marker were then scored for the coinheritance of muc and the unselected auxotrophic markers. The results thus obtained are shown in Table 7, and are based on the analysis of 100 recombinants for each selected marker.

These results clearly indicate that muc-22 and muc-23 are mutations in two distinct loci, both linked to cys-59 but on opposite sides of this marker. The gene arrangement consistent with the recombinant analysis data is illustrated in Figure 8.

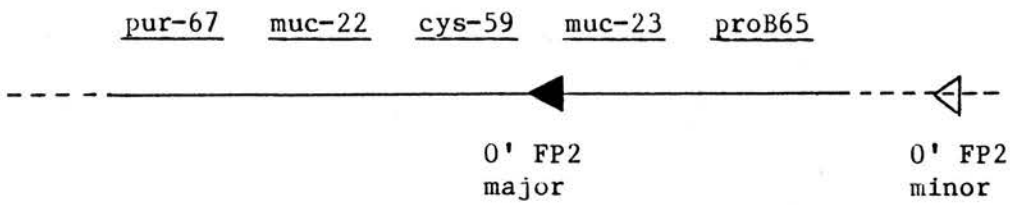


Fig. 8 Approximate locations of muc-22 and muc-23 relative to pur-67, cys-59 and proB65 based on results obtained in FP2-mediated plate matings.

At this point it was tempting to conclude that mutations occuring at either of these muc loci could be distinguished on the basis of their phenotypic characteristics, i.e. expression of alginate synthesis on MA. However, when similar crosses were performed using the mucoid donor strains PAO568 (muc-2) and PAO581

TABLE 7 Recombinant analysis following plate matings between the mucoid donors PA0578 and PA0579 and the non-mucoid (*muc*$^+$) recipient strain PA01042.

Donor	Selected marker	% Coinheritance of unselected marker			
		muc	*pro*$^+$	*cys*$^+$	*pur*$^+$
PA0578 (*muc-22*)	*proB65*$^+$	1		4	<1
	cys-59$^+$	34	33[a]		6[b]
	pur-67$^+$	10	2	5	
PA0579 (*muc-23*)	*proB65*$^+$	8		4	<1
	cys-59$^+$	38	35[c]		10[d]
	pur-67$^+$	2	2	4	

a 30% of these recombinants had coinherited *muc-22*

b 100% of these recombinants had coinherited *muc-22*

c 97% ofthese recombinants had coinherited *muc-23*

d 0% of these recombinants had coinherited *muc-23*

(muc-25), recombinant analysis revealed that like muc-22, both muc-2 and muc-25 are located between pur-67 and cys-59, despite the fact that PAO581 is a group 1 mucoid strain like PAO579. The lack of association between medium-dependent alginate expression and location of the muc mutation was further demonstrated when muc-45 and muc-52 (both giving rise to a group 1 phenotype), were mapped between cys-59 and pur-67 by R68.45-mediated plate matings.

1c) The mapping of muc mutations by transduction

To my knowledge, no selectable markers have been mapped between cys-59 and proB, so no further mapping of muc-23 has been possible, and the location of this mutation with respect to the major FP2 origin is provisional. However, it was possible to proceed further with the mapping of muc mutations located between pur-67 and cys-59, as the two catabolic loci pru (proline utilization) and oru (ornithine utilization) have recently been mapped in this region (Soldati et al, 1982).

Although it is possible to transduce most mucoid strains using F116L (PAO579 is an exception as it is apparently "resistant" to F116L), it is very difficult to obtain high titres of transducing phage propagated on mucoid strains by the plate harvest method, presumably because the phage receptors on the bacterial surface are masked by the exopolysaccharide when the strain is grown on solid medium. Consequently, the mapping of muc-22 by transduction required the isolation of a "suppressed" non-mucoid revertant of PAO578, i.e. a non-mucoid derivative in which alginate synthesis is switched off in some way by a second mutation unlinked to muc. Such

"suppressed" revertants are still able to transfer muc in matings, and are relatively common amongst non-mucoid revertants of PA0568, PA0578 and PA0579, but not PA0581.

Twelve non-mucoid revertants of PA0578 were crossed with PA01042, selecting for cys^+, and 20 recombinants from each scored for $Muc^{+/-}$ following transfer of partially purified colonies to PIA. $PA0578_{R11}$ was chosen as a typical "suppressed" revertant and used to propagate a transducing preparation of phage F116L. PA0954 (oru-292) and PA0964 (pru-354) were transduced using this F116L preparation and selection was made for Oru^+ and Pru^+ transductants. These transductants (200 in each case) were then scored for the coinheritance of muc-22. In this way, pru-354 and muc-22 were shown to be 20% cotransducible, whereas oru-292 and muc-22 were <0.5% cotransducible. Similarly, muc-2 was shown to be 49% cotransducible with pru-354, following the isolation of a "suppressed" non-mucoid revertant of PA0568, $PA0568_{R1}$. However, repeated attempts to isolate such a revertant of PA0581 were unsuccessful so it has not yet been possible to map muc-25 by transduction.

Further muc mutations (muc-37, muc-38, muc-39 and muc-40) were shown to be cotransducible with pru-354 with frequencies ranging from 32% to 46%. The strains carrying these mutations (PA06502, PA06503, PA06504 and PA06505) were all mucoid derivatives of PA0964, so it was possible to transduce these strains with F116L propagated on PA01, selecting for pru^+ and score for the coinheritance of muc^+.

1d) Demonstration of close linkage between muc-2 and muc-37

The cotransduction frequencies between pru-354 and the muc mutations muc-2 and muc-37 were 49% and 32% respectively, and strains carrying these muc mutations could be distinguished phenotypically on the basis of alginate synthesis or lack of it on P medium (see Table 6). To determine whether muc-2 and muc-37 could be separated genetically, F116L propagated on PA0568$_{R1}$ was used to transduce PA06502 selecting for Pru$^+$ transductants. Figure 9 indicates how non-mucoid transductants could arise if muc-2 and muc-37 are genetically separable with muc-2 closer to pru-354 than muc-22 (as suggested by the cotransduction frequencies).

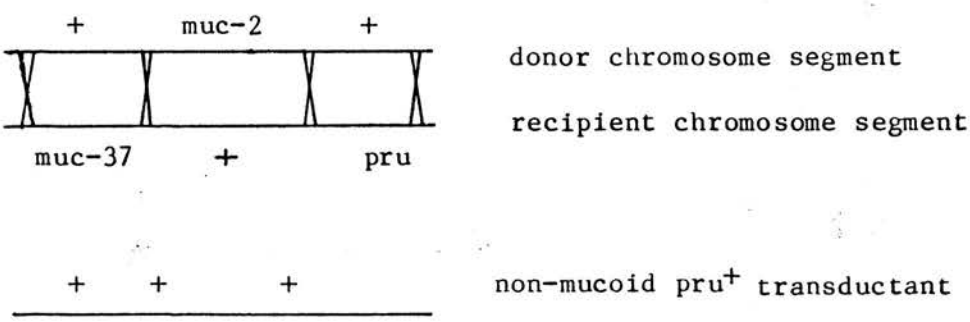


Fig. 9 The mechanism by which a muc$^+$ transductant could be obtained from a muc x muc cross.

However, the examination of 200 Pru$^+$ transductants revealed no non-mucoid colonies suggesting that muc-2 and muc-37 are mutations in the same locus or very closely linked loci.

1e) A-three-factor cross to determine the gene order for oru-292, pru-354 and muc-37

The plasmid R68.45 was transferred into PA0954, and the resulting strain used as donor in a plate mating with PA06502 selecting for Pru^+. In order to determine the relative locations of oru, pru and muc, 100 recombinants from this cross were partially purified on P medium and then replica plated to O medium and PIA to score for coinheritance of oru and muc^+.

Figure 10 shows the two possible gene arrangements for these 3 loci and Table 8 shows the results of recombinant analysis indicating the minimum numbers of cross-overs required to obtain each class of recombinant, based on the two gene arrangements.

a oru pru muc

b oru muc pru

Fig. 10 The two possible gene arrangements for oru, pru and muc.

Based on the assumption that a class of recombinant requiring four cross-overs would be extremely rare, and would certainly not be expected to occur with a frequency of 5%, the results shown in Table 8 are consistent with the gene order shown in Figure 10a, i.e. oru - pru - muc.

TABLE 8 The results of recombinant analysis following a plate mating between PA0954.R68.45 and PA06502

Selected markers	Unselected markers	Frequency (%)	Minimum number of cross-overs	
			a	b
pru-354	oru-292$^+$ muc-37$^+$	40	2	2
	oru-292$^+$ muc-37$^-$	1	2	2
	oru-292$^-$ muc-37$^+$	54	2	2
	oru-292$^-$ muc-37$^-$	5	2	4

Summary of results presented in Section 1

(i) There are at least 2 distinct loci on the PAO chromosome which are associated with the control of alginate synthesis. One of these loci lies between cys-59 and proB and only one mutation at this site (muc-23) has been mapped so far. A second muc locus (or cluster of closely linked loci is cotransducible with pru-354, distal to oru-292. Six independent muc mutations appear to be alleles at this locus, i.e. muc-2, muc-22, muc-37, muc-38, muc-39, and muc-40. Three additional mutations, muc-25, muc-45 and muc-52 are likely to be closely linked to this region on the basis of conjugational linkage to the flanking markers cys-59 and pur-67.

(ii) Mucoid strains display considerable heterogeneity on the basis of medium-dependent expression of alginate synthesis. However, the genetic basis of this heterogeneity is not apparent.

2a) The construction of the Rec$^-$ derivatives, PA06519 and PA06520 for use in the isolation of R'muc and R'muc$^+$ plasmids

The isolation and maintenance of R' plasmids derived from R68.45 are dependent on the availability of recA P. aeruginosa strains carrying the markers of interest (Holloway, 1978). As it was not possible to select directly for R' plasmids carrying muc$^{+/-}$, it was decided to select for R' pru$^+$ plasmids and screen these for the presence of muc$^+$ or the relevent muc allele (depending on the donor strain used). Hence it was necessary in the first instance to construct two recA recipient strains, PA06519 (pru-354, muc-37, rec-102) and PA06520 (pru-354, rec-102) by the series of steps to be described.

(i) A Pro$^+$ derivative of PA01042 was obtained following transduction with F116c propagated on PA01. This step was necessary to remove the proB65 marker leaving a strain with the genotype thr-9001, pur-67, cys-59 (PA06506).

(ii) It was necessary to construct donor derivatives of PA0964 and PA06502 so that pru-354 and muc-37 could be transferred into PA06506. Hence, Met$^-$ auxotrophs of these strains were isolated to provide a contraselective marker which did not interfere with Pru, and R68.45 was transferred into these derivatives to give PA0964 Met$^-$.R68.45 and PA06502 Met$^-$.R68.45.

(iii) PA0964 Met$^-$.R68.45 and PA06502 Met$^-$.R68.45 were plate mated with PA06506 selecting for the coinheritance of Pur$^+$ and Cys$^+$, since pru-354 and muc-37 lie between pur-67 and cys-59.

The recombinants thus obtained were checked for the inheritance of muc-37 and/or pru-354. In addition it was important to ensure that R68.45 was not present in the recombinants. The resultant strains at the end of this step had the genotypes pru-354 thr-9001 and pru-354, muc-37, thr-9001.

(iv) Früh et al (1983) have shown that rec-102 (a recA-like mutation) is 62% linked to thr-9001 in R68.45-mediated plate matings. Hence, the rec-102 donor strain PT066 R68.45 was crossed with the two strains constructed in step (iii), selecting for Thr^+ recombinants. Thirty such recombinants from each cross were then tested for the coinheritance of rec-102 on the basis of UV sensitivity and inability to form transductants despite retaining sensitivity to phage F116L. Once again, recombinants which had inherited R68.45 were not considered. Thus, PA06519 (pru-354, muc-37, rec-102) and PA06520 (pru-354, rec-102) were the resultant strains at the end of this procedure.

2b) The isolation of R'pru^+ plasmids

PA08 R68.45 was patch mated with PA06519 and plated on to P medium. After 3 days incubation at 37°C, 41 Pru^+ colonies had grown up and these were purified by streaking for single colonies on fresh P medium.

There are several possible mechanisms by which these colonies could have arisen: (i) R'pru^+ plasmids arising at a low frequency in the R68.45 donor could have transferred to PA06519 by

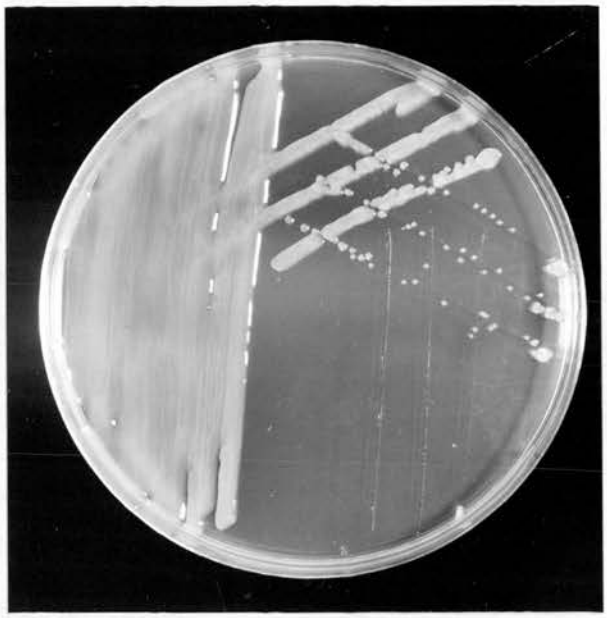
conjugation, thus producing a merodiploid. Such strains would express the antibiotic resistance pattern associated with R68.45 (CB^r, Tc^r, KM^r) and be able to transfer *pru*$^+$ at a high frequency to both a Rec^+ or Rec^- recipient. In addition, loss of plasmid markers from a merodiploid strain would be associated with loss of the Pru^+ characteristic. (ii) Rare chromosomal recombinants may have arisen if the *rec-102* mutation in PA06519 was leaky. Colonies resulting from this mechanism would not be able to transfer *pru*$^+$ at a high frequency and would not require the presence of the plasmid for continued expression of Pru^+. (iii) The colonies may be Pru^+ revertants of PA06519, in which case they would not be expected to carry the plasmid.

The 41 Pru^+ isolates were screened for CB^r by patching on to DSTA + 500μg ml^{-1} carbenicillin so that any Pru^+ revertants of PA06519 could be discarded. However, all were found to be CB^r. In 27 of the isolates, the Pru^+ characteristic was unstable as subculture on P medium gave rise to large and small colonies. The small colonies failed to grow on further subculture on fresh P medium, although they retained CB^r, suggesting that in these strains *pru*$^+$ was indeed carried on the plasmid, but was lost at a high frequency, rather than the whole plasmid being lost. These unstable isolates were discarded. The remaining 14 isolates grew well on further subculture to P medium and were screened for KM^r and Tc^r by patching on to DSTA + 500μg ml^{-1} kanamycin and DSTA + 100μg ml^{-1} tetracycline respectively, and scored for alginate production on these complete media (this characteristic could not be scored on P medium as PA06519 is non-mucoid on P medium). Nine of the 14 Pru^+

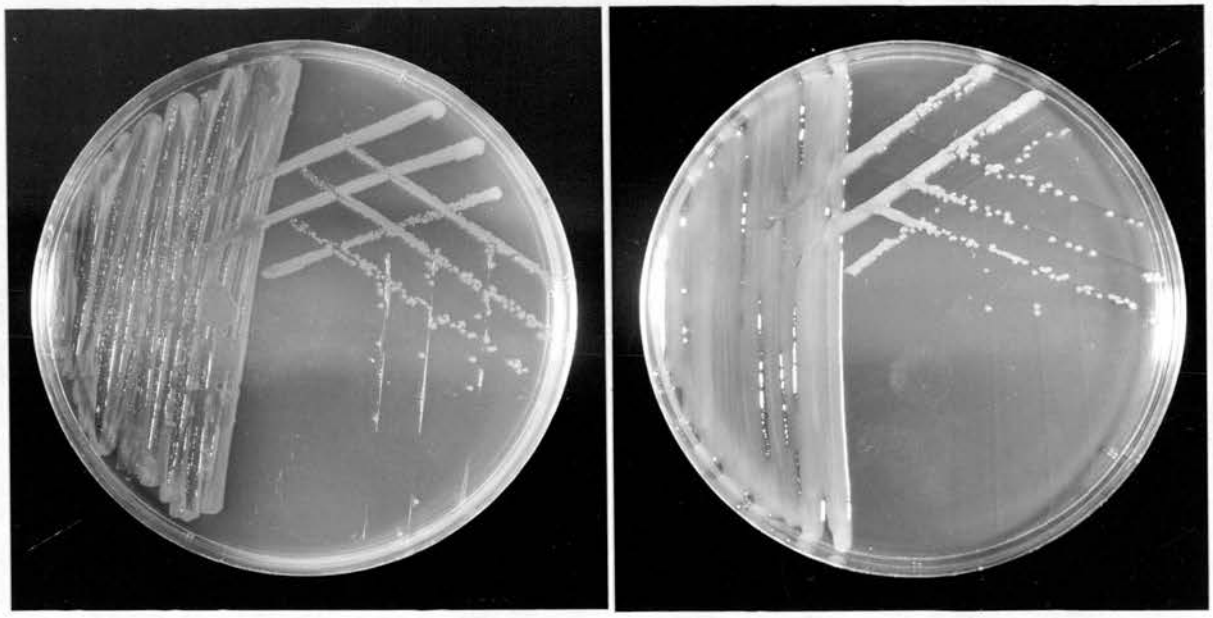
isolates were KM^r, Tc^r and remained mucoid, whereas the other five were non-mucoid, Tc^r but KM^s. One of the mucoid isolates and one of the non-mucoid isolates were tested for the ability to transfer $\underline{pru}^+$ at a high frequency to a RIF^r derivative of PA06519 (PA06519 RIF^r). Both were able to transfer $\underline{pru}^+$ at the same frequency as CB^r in a patch mating, so it was concluded that these strains contained $R'\underline{pru}^+$ plasmids and were designated PA06519 pJF4 (the non-mucoid isolate) and PA06519 pJF6 (the mucoid isolate). Figure 11 shows PA06519, PA06519 pJF4 and PA06519 pJF6 on PIA after 24h at 37°C.

2c) $\underline{pJF4\ carries\ muc^+\ in\ addition\ to\ pru^+}$

The non-mucoid phenotype of PA06519 pJF4 suggested that either (i) pJF4 carried $\underline{muc}^+$ in addition to $\underline{pru}^+$, and $\underline{muc}^+$ was dominant over $\underline{muc-37}$, or (ii) pJF4 had transferred from PA08 to a non-mucoid revertant of PA06519 during the patch mating (so pJF4 carried $\underline{pru}^+$ only). To distinguish between these two possibilities, a broth mating was performed between a 5h NB culture of PA06519 pJF4 and an 18h NB culture of PA06519 RIF^r. After $1\frac{1}{2}$h at 37°C, viable counts were determined of the CB^r and Pru^+ transconjugants (on DSTA + 500µg ml^{-1} carbenicillin, 250µg ml^{-1} rifampicin and P medium + 250µg ml^{-1} rifampicin respectively). In each case, the viable counts were 1×10^5 organisms ml^{-1}, and 99% of the transconjugants were non-mucoid, thus confirming that Pru^+ and Muc^+ were transferred together with CB^r. The viable count of the donor culture (estimated on P medium) was 7.8×10^7 organisms ml^{-1}, indicating the transfer frequency of pJF4 to a Rec^- recipient in a $1\frac{1}{2}$h broth mating was approximately 10^{-3} per donor. A similar transfer frequency was obtained whenthe



a



b c

Figure 11 The appearance of the mucoid Rec⁻ recipient strain PA06519 (a), and the same strain carrying R' plasmids pJF4 (b) and pJF6 (c), following 24h incubation at 37°C on PIA

same culture of PAO6519 pJF4 was broth mated with the Rec^+ mucoid recipient PAO6502 RIF^r. In this mating, 96% of the CB^r transconjugants were non-mucoid (i.e. Muc^+) and Pru^+, with the remaining 4% mucoid (Muc^-) and Pru^-, indicating that pJF4 is less stable in Rec^+ background than a Rec^- background.

2d) The association of Muc^+ and KM^s in pJF4

Viable counts of the 5h NB culture of PAO6519 pJF4 described in the previous section were also estimated on DSTA, DSTA + 500µg ml^{-1} kanamycin, DSTA + 500µg ml^{-1} carbenicillin and DSTA + 100µg ml^{-1} tetracycline, and compared with the viable count obtained on P medium. These results are shown in Table 9 and clearly show that PAO6519 pJF4 is KM^s but segregates mucoid and non-mucoid KM^r colonies at frequencies of approximately 10^{-6} and 10^{-5} respectively. The viable counts on DSTA, DSTA + carbenicillin, DSTA + tetracycline and P medium were not significantly different indicating that *pru*$^+$, *muc*$^+$, CB^r and Tc^r are stably maintained when PAO6519 pJF4 is cultured under the conditions described.

To determine the nature of the KM^r revertants, 25 mucoid and 25 non-mucoid colonies were tested for the other pJF4 markers. All colonies were CB^r and Tc^r, and all the non-mucoid colonies were Pru^+, but unable to transfer KM^r to PAO6519 RIF^r (despite being able to transfer all the pJF4 markers), suggesting that KM^r in these strains resulted from a chromosomal mutation. All the mucoid KM^r colonies were Pru^- indicating that the chromosomal markers had been lost from pJF4 (i.e. pJF4 had reverted to R68.45) in these strains.

TABLE 9 The viable counts of a 5h NB culture of PAO6519 pJF4 on different selective media

Medium	Viable Count (organisms ml^{-1})	
	Non-mucoid	Mucoid
DSTA	1.5×10^8	$<1.5 \times 10^7$
DSTA + carbenicillin	2.0×10^8	$<2.0 \times 10^7$
DSTA + tetracycline	3.3×10^7	$<3.3 \times 10^6$
DSTA + kanamycin	1.1×10^3	1.4×10^2
P	7.8×10^7	ND

ND = not determined

Thus it appears that the acquisition of the chromosomal DNA segment carrying *muc*$^+$ and *pru*$^+$ by R68.45, resulting in pJF4 (and the other R'*pru*$^+$*muc*$^+$ plasmids) was associated with "switching off" KM^r, perhaps due to the insertion of the chromosomal DNA into the *Km* gene. This mechanism of R' formation was apparently not operating when the R'*pru*$^+$ plasmids, e.g. pJF6, were derived.

2e) Additional chromosomal markers associated with pJF4

Markers in the vicinity of *muc* and *pru* on the PAO chromosome map include *oru*, *cys* and *pur*. To determine whether pJF4 carried the wild type alleles of any of these markers, broth matings were set up between PAO6519 pJF4 and PAO961 RIF^r (to test for *oru*$^+$) and PAO1042 RIF^r (to test for *cys*$^+$ and *pur*$^+$). Selection was made for CB^r transconjugants and 50 such colonies from each mating were scored for the coinheritance of the wild type allele of the relevant chromosomal marker. A high percentage coinheritance would indicate that pJF4 carried the wild type allele in question, and consequently, pJF4 was shown to carry *oru*$^+$, but not *cys*$^+$ or *pur*$^+$.

2f) The transfer of pJF4 to other mucoid PAO strains

To determine whether pJF4 could "switch off" alginate synthesis in PAO strains with *muc* mutations other than *muc-37*, the R' plasmid was transferred into PAO568, PAO578, PAO579, PAO581 and PAO6513 in broth matings followed by selection for CB^r transconjugants on DSTA + 500μg ml^{-1} carbenicillin , 250μg ml^{-1} streptomycin. On this medium, the transconjugants could be scored directly for alginate

production. To ensure that any effect on the expression of alginate synthesis was due to the merodiploid state of the muc locus, control crosses were set up between PAO6519 pJF6 and the mucoid recipients. All strains except PAO579 were rendered non-mucoid by pJF4, no strains became non-mucoid with the acquisition of pJF6.

Thus it appears that the plasmid determined muc^+ allele in pJF4 is dominant over muc-2, muc-22, muc-25, muc-45 and muc-37 (i.e. the muc mutations which are closely linked to pru), when those muc mutations are carried onthe chromosome. On the basis of the early work on the control of colanic acid synthesis in E. coli by Markovitz and colleagues (reviewed in Markovitz, 1977), this result was consistent with the hypothesis that muc^+ codes for a cytoplasmic repressor which is involved in the regulation of the alginate biosynthetic pathway.

Alginate synthesis in PAO579 is not switched off by pJF4, as this R' plasmid doesnot carry the $muc\text{-}23^+$ allele, and a double dose of the $muc\text{-}37^+$ gene product cannot apparently override the effect of the muc-23 mutation.

2g) Isolation of R' plasmids carrying muc-2, muc-22 and muc-45

In E. coli K12, a mutation at the capR locus leads to derepression of colanic acid synthesis. One particular mutation at this locus, capR6 is recessive to $capR^+$ in a merodiploid, regardless of whether the mutant gene is chromosomal or F' determined (Markovitz, 1977). However, a second mutation at the same locus, capR9, is dominant over the wild type allele when the former is F'

associated, i.e F'capR9/capR$^+$ merodiploid strains were mucoid, whereas F'capK$^+$/capR9 strain was non-mucoid.

To determine the allele dominance of muc-2, muc-22 and muc-45, R68.45 was first transferred into strains carrying these mutations (PAO6527, PAO6529 and PAO6513 respectively). PAO568 and PAO578 could not be used as suitable backgrounds for muc-2 and muc-22 in this experiment as they contained FP2. R'pru$^+$ plasmids were obtained from patch matings between the rec-102 non-mucoid recipient PAO6520, and each of the mucoid donor strains, and when streaked on to DSTA + 500μg ml^{-1} carbenicillin and incubated for 24h at 37^{o}C, a proportion of the R'pru$^+$ plasmids from each of the matings was mucoid, indicating that they carried the appropriate muc mutation which was dominant over the chromosomal muc$^+$. These R'plasmids were designated pJF54 (R'muc-22), pJF55 (R'muc-2) and pJF56 (R'muc-45). Figure 12 shows PAO6520, PAO6520 pJF54, PAO6520 pJF55 and PAO6520 pJF56 on PIA after 24h incubation at 37^{o}C. R'muc-45/muc$^+$ and R'muc-22/muc$^+$ are distinctly mucoid, whereas, the R'muc-2/muc$^+$ strain is only slightly mucoid. Each of these R' plasmids was KM^r and Tc^r.

2h) The transfer of pJF4 into mucoid P. aeruginosa isolates from CF patients

In PAO, pJF4 "switches off" alginate synthesis in mucoid strains with muc mutations linked to pru, suggesting that pJF4 could be used as a probe to determine whether an unknown muc mutation, e.g. in a clinical mucoid strain mapped in this region or elsewhere. Consequently, RIF^r mutants were isolated of the five clinical P. aeruginosa strains 492a, 1949, 2246, 2249 and 2263. These

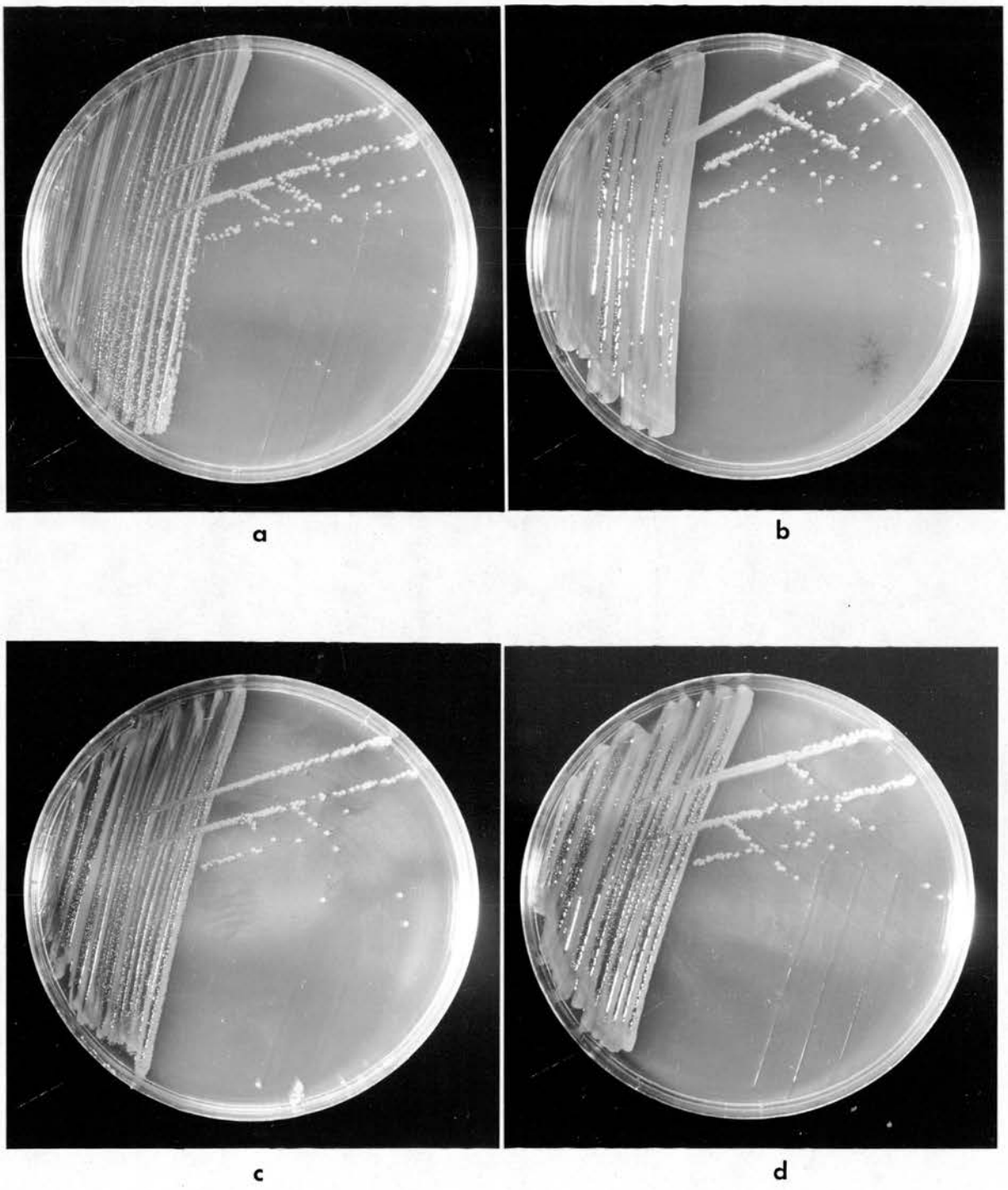


Figure 12 The appearance of the non-mucoid Rec$^-$ recipient strain PAO6520 (a) and the same strain carrying the R' plasmids pJF54 (b), pJF55 (c) and pJF56 (d), following 24h incubation at 37°C on PIA.

strains were mucoid isolates from different CF patients. pJF4 was transferred into the RIF^r derivatives in the usual way, selecting for CB^r transconjugants. In four of the five strains (492a RIF^r was the exception), the CB^r transconjugants were non-mucoid, suggesting that 1949, 2246, 2249 and 2263 have *muc* mutations which are linked to *pru*. It is not known whether the *muc* mutation in 492a is like *muc-23*, or is in a hitherto unmapped *muc* locus

Summary of the results presented in Section 2

(i) The construction of suitable Rec$^-$ strains for the isolation of R'*muc* and R'*muc*$^+$ plasmids is described.

(ii) Fourteen stable R'*pru*$^+$ plasmids were isolated following a patch mating between PAO8.R68.45 and the Rec$^-$ mucoid (*muc-37*) strain PAO6519. Five of these also carried *muc*$^+$. The plasmids chosen as representatives of the R'*pru*$^+$*muc*$^+$ and R'*pru*$^+$ classes were pJF4 and pJF6 respectively.

(iii) pJF4 (and the other R'*pru*$^+$*muc*$^+$ plasmids) were KM^s, unlike R68.45 and pJF6. Reversion to KM^r in pJF4 was associated with loss of *pru*$^+$ and *muc*$^+$.

(iv) In addition to *pru*$^+$ and *muc*$^+$, pJF4 also carries the wild type allele at the *oru* locus.

(v) In a merodiploid, the plasmid-determined *muc*$^+$ allele was dominant over the chromosomal mutations *muc-2*, *muc-22*, *muc-25* and *muc-45* in addition to *muc-37*.

(vi) Merodiploid strains carrying a wild type chromosomal allele and either muc-2, muc-22 or muc-45 as the plasmid-associated marker were slightly mucoid.

(vii) pJF4 "switched off" alginate synthesis in 4/5 clinical mucoid strains isolated from CF patients.

CHAPTER IX

GENETIC STUDIES ON ANTIBIOTIC HYPERSENSITIVITY IN P. AERUGINOSA

Bacterial strains. The bacterial strains used in this study are listed in Table 10. Donor strains carrying the plasmid R68.45 were constructed as described in Chapter VI, Materials and Methods.

1. Antibiotic Sensitivity of P. aeruginosa Strains 492a and 492c

It has previously been reported that 492c is more sensitive than 492a to the antibiotics carbenicillin, azlocillin, piperacillin, ticarcillin, methicillin, tetracyline and trimethoprim (Irvin et al, 1981).

Table 11 shows the MIC's of an extended range of antibiotics for 492a and 492c. To summarize these results, 492c is hypersensitive (>10 times more sensitive than 492a) to the β-lactam antibiotics carbenicillin, methicillin, flucloxacillin, mecillinam and cefuroxime, and the quinolone, naladixic acid. Increased sensitivity (4 to 8 times more sensitive than 492a) to trimethoprim, chloramphenicol and novobiocin was also noted for 492c. No difference in sensitivity between the two strains was observed for benzyl penicillin, ampicillin, cefoxitim, cephaloridime, rifampicin, streptomycin, gentamicin, tobramycin or kanamycin.

TABLE 10 Strains of *P. aeruginosa* used in the study of antibiotic hypersensitivity

Strain	Genotype/Description*	Reference/Origin
PAO1	Prototrophic, *chl-2*	Holloway, 1969
PAO2	*ser-3*	Isaac & Holloway, 1968
PAO4	*arg-47*, *pyrB52*	Holloway collection
PAO8	*met-28*, *ilv-202*, *str-1*	Isaac & Holloway, 1968
PAO38	*leu-38*	Stanisich & Holloway, 1969
PAO222	*met-28*, *trp-6*, *lys-12*, *his-4*, *ilv-226*, *pro-82*	Haas & Holloway, 1976
PAO505	*met-9001*, *amiE200*	Rella & Haas, 1982
PAO969	*proC130*	Rella & Haas, 1982
PAO6002	*met-9001*, *amiE200*, *nalB4*	Rella & Haas, 1982
PAO6006	*proC130*, *nalB9*	Rella & Haas, 1982
PAO6511	*proC130*, *blsA2*	Carbenicillin hypersensitive mutant of PAO969
PAO6524	*proC130*, *nalB4*	Constructed from PAO8 using F116L mediated transduction
PAO6526	*blsA2*	Pro^+ transductant of PAO6511 obtained using F116c
GMA037	*hisV5037*	Mee & Lee, 1967
492a	Clinical isolate, mucoid, prototrophic, *tspA1*	Irvin *et al*, 1981
492a Leu^-	Leu^- mutant of 492a	Isolated following EMS treatment

TABLE 10 (cont.)

Strain	Genotype/Description*	Reference/Origin
492c	Clinical isolate, mucoid, prototrophic, blsA1, tpsA1	Irvin et al, 1981
492c Leu$^-$	Leu$^-$ mutant of 492c	Isolated following EMS treatment
519c	Clinical isolate, mucoid, prototrophic, antiobic hypersensitive	This thesis
519c Leu$^-$	Leu$^-$ mutant of 519c	Isolated following EMS treatment
2358	Clinical isolate, non-mucoid, prototrophic, antibiotic hypersensitive	This thesis
2358 Leu$^-$	Leu$^-$ mutant of 2358	Isolated following EMS treatment
799	Prototrophic	Zimmerman, 1979
799 Leu$^-$	Leu$^-$ mutant of 799	Isolated following EMS treatment
799/61	Prototrophic, antibiotic hypersensitive	Zimmerman, 1979
PAJ1	tpsA1, blsA1, str-1	CEF^{s}TP^{s}Met$^+$Ilv$^+$ recombinant from cross 492c Leu$^-$ R68.45 x PA08
PAJ2	blsA1, str-1	CEF^{s}TP^{s}Met$^+$Ilv$^+$ recombinant from cross 492c Leu$^-$ R68.45 x PA08
PAJ3	tpsA1, str-1	CEF^{r}TP^{s}Met$^+$Ilv$^+$ recombinant from cross 492c Leu$^-$ R68.45 x PA08
PAJ5	met-28, tpsA1, str-1	CEF^{r}TP^{s}Ilv$^+$ recombinant from cross 492c Leu$^-$ R68.45 x PA08

TABLE 10 (cont.)

Strain	Genotype/Description*	Reference/Origin
PAJ6	ilv-202, tpsA1, str-1	$Met^{+}Ilv^{-}$ transductant of PAJ5 constructed using F116c.PAO8 Met^{+}
PAZ1	met-28, trp-6, lys-12, his-4 ilv-226, blsB3	$CEF^{S}Pro^{+}$ recombinant from cross 61 Leu^{-} R68.45 x PAO222
PAZ2	trp-6, lys-12, his-4, ilv-226, pro-82, blsC4	$CEF^{S}Met^{+}$ recombinant from cross 61 Leu^{-} R68.45 x PAO222
PAZ3	lys-12, his-4, ilv-226, pro-82, blsC4	$CEF^{S}Met^{+}Trp^{+}$ recombinant from cross 61 Leu^{-} R68.45 x PAO222
PAZ4	lys-12, his-4, ilv-226, blsC4, blsB3	$RIF^{S}Pro^{+}$ transductant of PAZ3 constructed using F116c.PAZ1

* The genotype symbols are the same as those defined in Fig. 2 bls designates sensitivity to β-lactam antibiotics, tps, sensitivity to trimethoprim, naladixic acid and novobiocin. CEF^{S} is defined as sensitive to 100μg cefuroxime ml^{-1}, TP^{S} is defined as sensitive to 100μg trimethoprim ml^{-1}, RIF^{S} is defined as sensitive to 50μg rifampicin ml^{-1}.

TABLE 11 Antibiotic sensitivities of *P. aeruginosa* strains 492a and 492c

Antibiotic	MIC ($\mu g\ ml^{-1}$) Strain 492a	Strain 492c
Carbenicillin	80	0.6
Methicillin	>500	10
Benzyl penicillin	500	500
Ampicillin	100	100
Flucloxacillin	>500	50
Mecillinam	500	10
Cefuroxime	>500	10
Cefoxitin	400	400
Cephaloridine	500	500
Chloramphenicol	40	10
Tetracycline	10	6
Trimethoprim	40	5
Naladixic acid	60	2
Novobiocin	>500	100
Rifampicin	40	40
Streptomycin	40	40
Gentamicin	0.6	0.6
Tobramycin	0.4	0.4
Kanamycin	60	60

2. The Mapping of Loci Associated with Antibiotic Hypersensitivity in 492c

Figure 13 indicates the locations of genetic markers relevant to this chapter on the PAO chromosome map.

2a) The transfer of two genetic determinants associated with antibiotic hypersensitivity from 492c to PAO

A donor derivative of 492c was prepared by first isolating an auxotrophic mutant (Leu^-), to provide a means of contraselection, and then transferring R68.45 into this mutant, thus giving 492c Leu^- R68.45.

Plate matings were performed between 492c Leu^- R68.45 and PAO222 (a recipient strain with multiple auxotrophic markers). One hundred recombinants for each marker were scored for coinheritance of sensitivity to cefuroxime (CEF^S) by spotting the purified recombinants onto DSTA + 100µg cefuroxime ml^{-1} (the MIC of cefuroxime for PAO222 was 200µg ml^{-1}). Increased sensitivity to cefuroxime rather than carbenicillin was scored initially to avoid any problems associated with the inheritance of R68.45 by some of the recombinants (R68.45 codes for the TEM-2 β-lactamase which is active against carbenicillin, but not cefuroxime). Recombinant analysis revealed that 56% of the *met-28*$^+$ recombinants were CEF^S, as were 20% of the *trp-6*$^+$ recombinants. Less than 1% of the recombinants for the other markers, i.e. *ilv-226*, *his-4*, *lys-12* and *pro-82* had coinherited this characteristic.

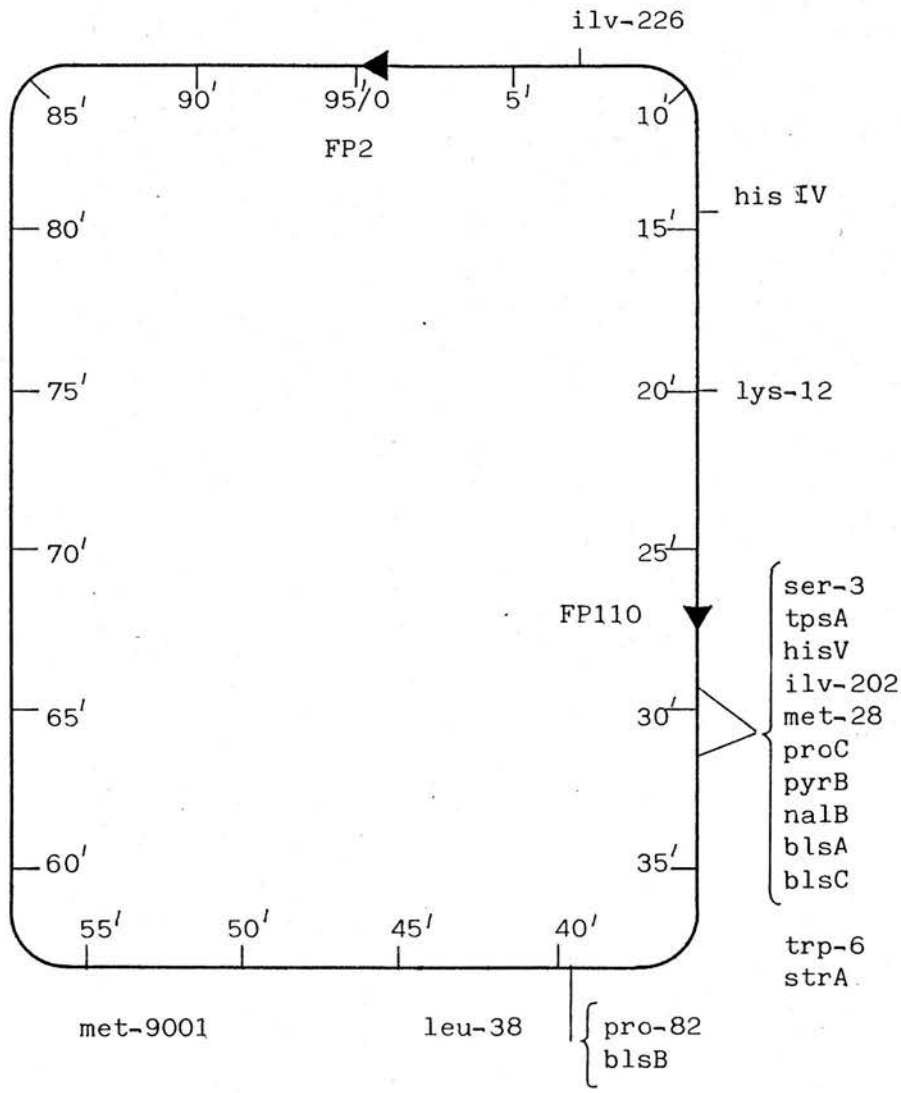


Figure 13 The P. aeruginosa PAO chromosome map showing the locations of markers relevant to this chapter. Marker designations are the same as for Fig.2.

On the basis of this result, a plate mating was performed between 492c Leu$^-$ R68.45 and PA08 (met-28, ilv-202) in order to precisely map the locus associated with CEF^s. Following selection for both auxotrophic markers, recombinant analysis was performed as before, except that recombinants were also scored for sensitivity to trimethoprim (TP^s) on DSTA + 100μg trimethoprim ml^{-1} (the MIC of trimethoprim for PA08 is 200μg ml^{-1}). The results of this recombinant analysis are presented in Table 12, and clearly show that CEF^s and TP^s do not always co-segregate. All CEF^s recombinants were TP^s, however 7% of the TP^s colonies were resistant to 100μg cefuroxime ml^{-1} (CEF^r).

Further characterization of the 3 classes of recombinants thus identified, i.e. CEF^{s}TP^s, CEF^{r}TP^s and CEF^{r}TP^r, was performed by determining the MIC's of all the antibiotics to which 492c had been shown to be more sensitive in this study (carbenicillin, methicillin, flucloxacillin, cefuroxime, mecillinam, chloramphenicol, trimethoprim, naladixic acid and novobiocin). On this basis, the CEF^{r}TP^r recombinants (3 tested) were indistinguishable from PA08. The CEF^{s}TP^s recombinants (8 tested) could be divided into 2 further groups on the basis of the MIC's of trimethoprim, naladixic acid and novobiocin. Three such recombinants were hypersensitive to these antibiotics with relative MIC's (i.e. $\frac{\text{MIC for recombinant}}{\text{MIC for PA08}}$ of 1/20, 1/16 and 1/50 respectively. The other 5 CEF^{s}TP^s recombinants were moderately sensitive with relative MIC's of 3/10 for trimethoprim, 1/4 for naladixic acid, and hypersensitive

TABLE 12 Results of recombinant analysis following plate matings between 492c Leu$^-$ R68.45 and PA08

Selected Marker	Percentage coinheritance of unselected character			
	CEF^s	TP^s	Met$^+$	Ilv$^+$
met-28$^+$	80	82	-	77
ilv-202$^+$	75	88	87	-

to novobiocin (relative MIC = 1/10). All 8 recombinants were equally sensitive to the β-lactam antibiotics, and chloramphenicol, and with the exception of cefuroxime (relative MIC = 1/4), were as sensitive as 492c.

The third class of recombinant, $CEF^{r}TP^{s}$ (5 tested) was moderately sensitive to trimethoprim and naladixic acid with relative MIC's of 3/10 and 1/4 respectively, and hypersensitive to novobiocin (relative MIC = 1/50).

From these results, it was concluded that the antibiotic hypersensitivity of 492c is associated with two separate genetic determinants, both linked to met-28 and ilv-202, and transferable to PAO. The first of these determinants (blsA1) encodes for hypersensitivity to carbenicillin, methicillin, flucloxacillin, mecillinam and novobiocin, and increased sensitivity to chloramphenicol, trimethoprim and naladixic acid. The second determinant (tpsA1) also encodes for hypersensitivity to novobiocin and increased sensitivity to trimethoprim and naladixic acid, but has no effect on sensitivity to β-lactams or chloramphenicol. Table 13 presents the MIC's of the relevent antibiotics for PA08 and three typical prototrophic recombinant strains PAJ1 (blsA1, tpsA1), PAJ2 (blsA1) and PAJ3 (tpsA1), obtained from the mating between 492c Leu⁻ R68.45 and PA08. Figure 14 indicates the approximate locations of blsA and tpsA relative to met-28 and ilv-202, based on the results of that plate mating (shown in Table 12).

TABLE 13 Antibiotic sensitivities of PA08 and recombinant strains PAJ1, PAJ2 and PAJ3, obtained following a plate mating between 492c Leu⁻ R68.45 and PA08

Antibiotic	Strain...	MIC ($\mu g\ ml^{-1}$) PA08	PAJ1 (*blsA1*, *tpsA1*)	PAJ2 (*blsA1*)	PAJ3 (*tpsA1*)
Carbenicillin		40	0.4	0.4	40
Methicillin		500	10	10	500
Flucloxacillin		>500	50	50	>500
Mecillinam		500	10	10	500
Cefuroxime		400	100	100	100
Chloramphenicol		40	20	20	40
Trimethoprim		200	10	60	60
Naladixic acid		80	5	20	20
Novobiocin		500	10	50	10

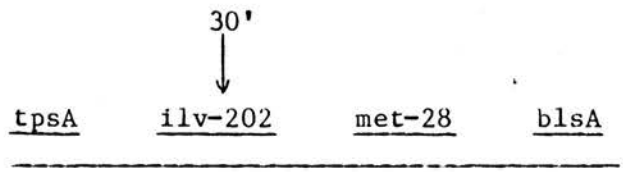


Fig. 14 Approximate locations of blsA and tpsA on the basis of results presented in Table 11.

2b) Evidence for tpsA1 in 492a

A comparison between the results presented in Tables 10 and 12 indicates that PA08 and 492a are similar with respect to antibiotic MIC's, with the exception of trimethoprim. 492a is inhibited by 40µg trimethoprim ml^{-1}, whereas the MIC for PA08 is 200µg ml^{-1} (a 5-fold difference). In addition, the increased sensitivity to trimethoprim of 492c compared with 492a (relative MIC = 1/8) is of the same order as the difference in MIC between PAJ1 and PAJ3 (relative MIC = 1/6). 492a is also more sensitive to naladixic acid than PA08. Despite the fact that 492c is resistant to novobiocin, it was proposed that the tpsA1 allele might be present in both 492a and 492c, and that the antibiotic hypersensitivity associated with the latter strain can be explained on the basis of a single mutation at the blsA locus (i.e. blsA1).

To test this hypothesis, a donor derivative of 492a (492a Leu^- R68.45) was constructed as described for 492c. 492a Leu^- R68.45 was subsequently plate mated with PA08, selecting for Met^+ and Ilv^+ recombinants. The results of recombinant analysis (performed as described for the mating 492c Leu^- R68.45 x PA08) were consistent with the hypothesis, i.e. 492a Leu^- R68.45 transferred TP^S to PA08,

but not CEF^S, and the gene associated with TP^S mapped in the same region as tpsA1.

2c) The association between tpsA1, serum sensitivity and PA

Both 492a and 492c are serum sensitive and PA, so it was thought that the presence of tpsA1 in these strains might be associated with one or both of these characteristics. To test this possibility, the recombinant strains PAJ1, PAJ2 and PAJ3, along with PA08 as control, were tested for serum sensitivity and agglutinability with multiple typing sera. However, all four strains were serum resistant and retained the type II serotype characteristic of PAO indicating that at least when transferred into PA08, tpsA1 was not associated with these characteristics.

2d) Mapping of blsA and tpsA by transduction

From the recombinant analysis following plate matings, it was possible to assign the chromosomal locations illustrated in Figure 14 for blsA and tpsA. However, to map these loci with respect to other markers in the 30 min region, transductional analysis was performed.

In the case of blsA1, a preparation of phage F116L was grown on PAJ2, and used to transduce recipient strains carrying the auxotrophic markers met-28 (PA08), ilv-202 (PA08), proC130 (PA0969) and pyrB52 (PA04). Selection was made for these markers and cotransduction of blsA1 scored on DSTA + 5μg carbenicillin ml^{-1}.

However, in the case of tpsA1, transducing preparations of F116L could not be propagated on PAJ3 or any other recombinants containing this marker, as they did not appear to support vegetative growth of F116L. Evidence that tpsA1 strains retained the receptors for the phage was provided by the observation that such strains were good recipients in F116L-mediated transductions. Consequently, the transductional mapping of tpsA1 was performed following the construction of an ilv-202 tpsA1 derivative, PAJ6, via the intermediate strain PAJ5 (see Table 9 for genotype). PAJ6 was used as recipient in transductions mediated by F116L propagated on either PAO1 (prototrophic), PAO2 (ser-3) or GMA 037 (his V5037). In each case, selection was made for Ilv^+ transductants and these were scored for the coinheritance of tpsA1 (on DSTA + 100µg trimethoprim ml^{-1}), and where appropriate, the unselected auxotrophic marker. Figure 15 shows the locations thus obtained for blsA and tpsA.

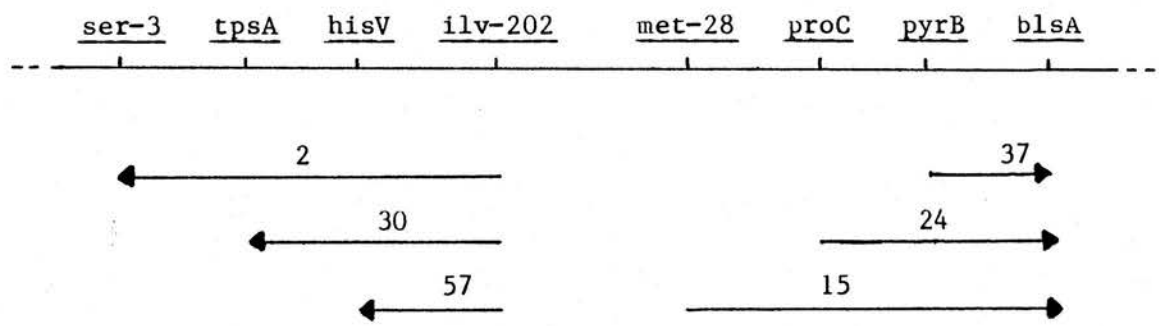


Fig. 15 Map locations obtained for blsA and tpsA following transductional analysis. Values above arrows indicate percentage cotransduction using phage F116L. Arrowheads point to the unselected marker.

3. The Mapping of Loci Associated with Antibiotic Hypersensitivity in Clinical P. aeruginosa Isolates 519c and 2358

P. aeruginosa strains 519c and 2358 were isolated from different CF patients. They express similar antibiograms to 492c, but are different pyocin types from 492c and from each other. 519c is mucoid and 2358 is non-mucoid.

To determine whether the genetic basis of antibiotic hypersensitivity was similar in these strains to that demonstrated in 492c, donor derivatives were constructed (519c Leu$^-$ R68.45 and 2358 Leu$^-$ R68.45) as described for 492c, and these strains were plate mated with PA08, selecting for Met$^+$ and Ilv$^+$ recombinants. Analysis of 100 of each recombinant class was performed by scoring for the coinheritance of CEF^S and TP^S (as with 492c). These results are shown in Table 14, and clearly indicate that the donor derivatives of 519c and 2358 can transfer CEF^S and TP^S to PA08, and that the loci associated with these characteristics are closely linked to met-28 and ilv-202 and do not always cosegregate, i.e. the results were very similar to those obtained for 492c.

4a) Isolation of a blsA Mutant of PA0969

If 492c arose from 492a by a single mutation at the blsA locus, it was proposed that a similar blsA mutant could be isolated from a PAO strain.

The strain chosen for the isolation of this mutant was PA0969 (proC130) and so following mutagenesis with EMS, approximately 5000 potential mutant colonies were screened on DSTA + 5µg carbenicillin

TABLE 14 Results of recombinant analysis following plate matings between the donor derivatives 519c Leu⁻ R68.45, 2358 Leu⁻ R68.45 and recipient PA08

Donor	Selected marker	Percentage coinheritance of unselected character	
		CEF^s	TP^s
519c Leu⁻ R68.45	met-28⁺	79	98
	ilv-202⁺	62	87
2358 Leu⁻ R68.45	met-28⁺	84	90
	ilv-202⁺	74	77

ml^{-1}. Only one such colony was sensitive to this concentration of carbenicillin, and fortuitously, the mutation responsible for this increased sensitivity was 33% cotransducible with *proC*, and 44% cotransducible with *pyrB* using F116L. This mutant strain was designated PA06511 (*bls-2*) and was found to be more sensitive than PA0969 to cefuroxime, trimethoprim, naladixic acid, novobiocin and chloramphenicol.

A comparison of the percentage cotransduction between *blsA1* and *proC* (24%), and *bls-2* and *proC* (33%), suggested that *bls-2* was closer to *proC* than *blsA1*. However, when F116L.PAJ2 was used to transduce PA06511 selecting for $proC^+$, <0.3% of these transductants were able to grow in the presence of 5µg carbenicillin ml^-, indicating that *blsA1* and *bls-2* are very closely linked. Consequently *bls-2* was renamed *blsA2*.

4b) The relationship between blsA and nalB

Rella & Haas (1982) have described a class of mutants (*nalB*) in *P. aeruginosa* PAO which show increased resistance to naladixic acid, novobiocin, carbenicillin and several additional β-lactams, e.g. piperacillin, azlocillin, ticarcillin and cefsulodin.

The existence of such mutants suggested that the antibiotics to which *nalB* mutants are more resistant may be the same ones to which *blsA* mutants are more sensitive. To test this, the MIC's of cefuroxime, trimethoprim, methicillin, chloramphenicol, ampicillin and benzyl penicillin were determined for two *nalB* mutants PA06002 (*nalB4*) and PA06006 (*nalB9*) and their respective parent strains

PA0565 and PA0969. As expected the nalB strains showed increased resistance to the first four antibiotics, with the following relative MIC's (with respect to the parent strains): cefuroxime, 3; trimethoprim, 6; methicillin, 5; chloramphenicol, 10. No increased resistance to ampicillin or benzyl penicillin was observed for the nalB strains.

The nalB locus has been mapped at 32 min on the PAO chromosome, and is 32% cotransducible with pyrB, using phage G101 (Rella & Haas, 1982). To determine the location of blsA with respect to nalB, reciprocal transductions were performed using F116L propagated on proC$^+$ strains carrying mutations at these loci, i.e. PA06002 (nalB4) and PA06526 (blsA2), and the equivalent proC recipients, i.e. PA06511 (blsA2) and PA06524 (nalB). The results of these transductions are presented in Table 15, and indicate that blsA and nalB are separate, but closely linked loci, with nalB mapping between proC and blsA.

5. The Mapping of Loci Associated with Antibiotic Hypersensitivity in 799/61

5a) The transfer of two determinants associated with CEF^S into PAO

The mutant strain 799/61 is highly sensitive to a large number of different antibiotics (Zimmerman, 1979), including those to which 492c is hypersensitive, and in addition ampicillin, cefoxitin, benzyl penicillin, cephaloridine and rifampicin (to which 492c is not unduly susceptible).

TABLE 15 Results of F116L-mediated transductions to determine the relationship between *nalB* and *blsA*

F116L grown on	Recipient (*proC*)	Percentage cotransdcution with *proC*$^{+}$*		
		blsA2	*nalB4*	wild type
PAO1 (wild type)	PAO6511 (*blsA2*)	-	-	33
"	PAO6524 (*nalB*)	-	-	43
PAO6002 (*nalB*)	PAO6511 (*blsA2*)	-	27	<0.5
PAO6526 (*blsA2*)	PAO6524 (*nalB*)	19	-	3

* 200 *proC*$^{+}$ transductants were scored for the coinheritance of *nalB4*$^{+/-}$ and *blsA2*$^{+/-}$ where appropriate, on DSTA + 50μg and 5μg carbenicillin ml^{-1}, respectively

In order to compare the genetic basis of antibiotic hypersensitivity in 799/61 with that demonstrated in 492c, 519c, 2358 and PAO6511, a donor derivative of 799/61 (61 Leu$^-$ R68.45) was constructed. This was performed in the same way as for the clinical isolates, however, selection for transfer of R68.45 into 61 Leu$^-$, following a broth mating, was made on MA + leucine + 50μg carbenicillin ml^{-1}, as no transconjugants were obtained when selection was made on the same medium supplemented with 500μg carbenicillin ml^{-1} (the concentration used to select for R68.45 transconjugants of 492c Leu$^-$, 519c Leu$^-$ and 2358 Leu$^-$).

Plate matings were performed between 61 Leu$^-$ R68.45 and PAO222, selecting for all the recipient auxotrophic markers. As only small numbers of recombinants were obtained (6 to 36 per marker), all were scored for the coinheritance of CEF^s. None of the recombinants selected for the inheritance of *ilv-226*$^+$, *his-4*$^+$ or *lys-12*$^+$ had coinherited CEF^s, whereas 2/13 *met-28*$^+$, 1/32 *trp-6*$^+$ and 17/36 *pro-82*$^+$ recombinants had coinherited this characteristic. A representative of the *pro*$^+$ CEF^s recombinants was designated PAZ1, a *met*$^+$ CEF^s representative was designated PAZ2, and the *trp*$^+$ CEF^s strain, PAZ3 (this strain was also *met*$^+$).

Similar matings performed between a donor derivative of 799 and PAO222 did not produce any CEF^s recombinants.

When R68.45 was transferred into PAZ3 and the resultant donor crossed with PAO8, 93% of the *met-28*$^+$ recombinants and 82% of the *ilv-202*$^+$ recombinants had coinherited CEF^s, suggesting a location for this determinant close to *blsA*.

An initial screening of the antibiotic sensitivities of PAZ1, PAZ2 and PAZ3 suggested that while PAZ2 and PAZ3 had probably inherited the same determinant which rendered the strains hypersensitive to carbenicillin and more sensitive to cefuroxime, methicillin, mecillinam and novobiocin, PAZ1 had either inherited an additional antibiotic hypersensitive determinant or a completely different one. This conclusion was based on the finding that PAZ1 was hypersensitive to naladixic acid, cefoxitin and rifampicin, in addition to carbenicillin, and was more sensitive than PAZ2 and PAZ3 to cefuroxime, methicillin, mecillinam and novobiocin.

5b) Mapping bls-3 and bls-4 by F116L-mediated transduction

It was possible to confirm that PAZ1 and PAZ3 carried different determinants associated with antibiotic hypersensitivity (initially designated bls-3 and bls-4) using transductional analysis. Transducing preparations of F116L were propagated on PAZ1 and PAZ3. F116L.PAZ1 was used to transduce PA0222 (selecting for pro-82$^+$) and PA038 (selecting for leu-38$^+$). F116L.PAZ3 was used to transduce PA0969 (selecting for proC$^+$) and PA08 (selecting for met-28$^+$). One hundred transductants for each selected marker were scored for the coinheritance of the appropriate bls determinant on DSTA + 5µg carbenicillin ml^{-1}. Results indicated that bls-3 is 40% cotransducible with pro-82 (at 40 min) and <1% cotransducible with leu-38. Thus, bls-3 was renamed blsB3 as it is obviously a different locus from blsA. In contrast, bls-4 was 19% cotransducible with proC and 9% cotransducible with met-28 indicating a location very close to blsA. However, blsA and bls-4

were shown to be distinct loci by transducing PAO6511 (proC130, blsA2) with F116L.PAZ3. Selection was made for $proC^+$ transductants, and 100 such transductants scored on DSTA + 5μg carbenicillin ml^{-1}. Consequently 4% of the transductants were able to grow on this medium and shown to be wild type with respect to carbenicillin sensitivity. Thus, bls-4 was designated blsC4.

These results are consistent with the gene arrangement illustrated in Figure 16.

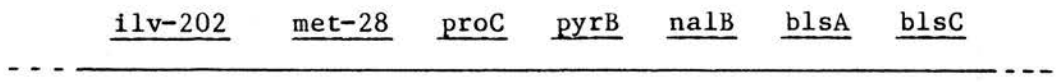


Fig. 16 Location of blsC relative to blsA and other markers in the 30 min region.

Evidence that the antibiotic hypersensitivity associated with PAZ1 was due to blsB3 and not a combination of blsB3 and blsC4 was obtained by screening the antibiotic sensitivities of a number of pro^+blsB3 transductants of PAO222. These were found to be the same as for PAZ1.

A derivative of PAZ3 carrying blsB3 in addition to blsC4 (PAZ4) was constructed using F116c propagated on PAZ1 to transduce PAZ3 selecting for $pro\text{-}82^+$. One hundred of these transductants were scored for coinheritance of blsB3 on DSTA + 50μg rifampicin ml^{-1}. PAZ4 was a representative of the rifampicin sensitive transductants.

5c) Antibiotic sensitivity associated with blsB3 and blsC4

Table 16 shows the MIC's of 14 antibiotics (to which 799/61 is hypersensitive) for PAO222, PAZ1, PAZ3 and PAZ4. With the exception of cephaloridine, PAZ4 (blsB3, blsC4) is extremely sensitive to all of these antibiotics.

The two genetic determinants blsB3 and blsC4 may or may not be responsible for the total antibiotic hypersensitivity of 799/61, however, when transferred into PAO222, the combination of these two mutations significantly lowers the MIC's of at least 13 antibiotics for this strain.

Summary of results presented in this chapter

1. A comparison of the antibiotic MIC's for the related clinical P. aeruginosa isolates 492a and 492c revealed that 492c is hypersensitive (>10 times more sensitive than 492a) to the antibiotics carbenicillin, methicillin, flucloxacillin, mecillinam, cefuroxime, and naladixic acid, and considerably more sensitive (4-8 times more sensitive than 492a) to trimethoprim, chloramphenicol and novobiocin. The MIC's of benzyl penicillin, ampicillin, cefoxitin, cephaloridine, rifampicin, streptomycin, gentamicin, tobramycin and kanamycin were identical for the two strains.

2. Two genetic determinants associated with the antibiotic hypersensitivity of 492c, were identified and mapped in the 30 min region of the chromosome, following plate matings between a

TABLE 16 Antibiotic sensitivities of PAO222, PAZ1, PAZ3 and PAZ4

Antibiotic	Strain...	MIC ($\mu g\ ml^{-1}$) PAO222	PAZ1 (*blsB3*)	PAZ3 (*blsC4*)	PAZ4 (*blsB3*, *blsC4*)
Carbenicillin		20	0.4	0.6	$\leqslant$0.2
Cefuroxime		200	10	40	5
Cefoxitin		400	20	400	$\leqslant$10
Cephaloridine		>600	600	>600	600
Methicillin		400	10	50	$\leqslant$1
Mecillinam		600	50	100	10
Flucloxacillin		>600	100	>600	50
Ampicillin		100	50	100	5
Benzyl Penicillin		600	200	400	50
Trimethoprim		>400	60	>400	20
Naladixic acid		400	40	400	20
Novobiocin		400	50	100	$\leqslant$10
Rifampicin		20	$\leqslant$1	20	$\leqslant$1
Chloramphenicol		>100	20	>100	20

donor derivative of 492c (492c Leu⁻ R68.45) and PA08. These determinants were designated blsA1 and tpsA1.

3. The tpsA1 determinant (associated with increased sensitivity to trimethoprim, naladixic acid and novobiocin) was also identified in 492a.

4. tpsA1 did not appear to be associated with serum sensitivity and PA which are characteristic of both 492a and 492c.

5. Transductional mapping using F116L located tpsA between the markers ser-3 and hisV, and blsA distal to pyrB, in the 30 min region of the chromosome.

6. The genetic basis of antibiotic hypersensitivity in the unrelated clinical isolates 519c and 2358 appeared to be very similar to that demonstrated for 492c.

7. A blsA mutant of PA0969 was isolated following EMS mutagenesis.

8. Transductional analysis revealed that blsA and nalB are closely linked, but separate loci.

9. A donor derivative of the Zimmerman hypersensitive mutant 799/61 was constructed, and used to transfer two determinants associated antibiotic hypersensitivity into PA0222.

10. One of these determinants, blsB3 was cotransducible with pro-82 and was associated with hypersensitivity to β-lactams, trimethoprim, naladixic acid, novobiocin, rifampicin and chloramphenicol.

11. The second determinant *blsC4* was closely linked, but distinct from *blsA* and gave rise to a similar phenotype as that associated with *blsA*.

DISCUSSION

CHAPTER X

DISCUSSION

It was stated in Chapter 1 to this thesis that P. aeruginosa is a catabolically versatile organism, capable of thriving in a wide variety of ecological niches, and can be considered a saprophyte, commensal, plant and animal pathogen.

What is it about this species that renders it so versatile? It is likely that genetic make-up is ultimately responsible.

The ability of P. aeruginosa to colonize, and express pathogenicity in one particular environment, i.e. the respiratory tract of the CF patient, is providing a challenging topic of research for workers of various backgrounds, ranging from clinicians and physiologists, to bacteriologists, geneticists and biochemists. Clinicians are approaching the subject from the point of view of the patient, by considering the pathological role of P. aeruginosa in the lung, and attempting to develop an effective therapeutic regime. Determination of the host and bacterial factors responsible for the early colonization of the respiratory tract by P. aeruginosa, in this particular patient group, is of interest to both clinicians and scientists alike, and has obvious implications for the prevention of subsequent pulmonary infection. However, from the point of view of microbial genetics, the growth and adaptation of P. aeruginosa in the potentially hostile environment of the CF respiratory tract, and the emergence of certain unusual characteristics which may or may

not contribute directly to pathogenesis, continues to provide an intriguing topic of study.

The main aim of this thesis was to apply classical genetic techniques to the analysis of the mechanisms associated with the emergence of two of these characteristics, i.e. alginate synthesis and antibiotic hypersensitivity, which are common in P. aeruginosa isolates from the sputa of CF patients suffering from chronic, debilitating pulmonary infection.

It could be argued that the bacteria isolated from sputum do not provide a good sample of the population present at the site of infection. However, Thomassen et al (1984) have recently demonstrated that the bacterial populations isolated from sputum and thoracotomy specimens from CF patients are indeed comparable.

Before considering the possible clinical implications of the results presented in this thesis, and because of the three different areas of research represented, I shall discuss these three independently with particular reference to current knowledge in the fields of (i) P. aeruginosa typing, (ii) alginate synthesis in mucoid P. aeruginosa, and (iii) intrinsic antibiotic resistance.

The application of pyocin typing to P. aeruginosa epidemiology

Revision of the Govan & Gillies pyocin typing method was undertaken to improve the application of this useful epidemiological tool for studies involving mucoid strains and multiple isolates from single specimens.

The spotting method has succeeded in shortening the time necessary to obtain a typing result from 48h to 24h, which although not comparable with the speed of serotyping, is nevertheless a significant improvement.

Application of the producer strains by means of a multiple inoculator, and elimination of the necessity to scrape off producer strain growth prior to adding the indicators, has meant a significant reduction in work load required for pyocin typing, particularly when large numbers of strains are involved.

The epidemiological value of distinguishing between S-pyocin production and R- or F-pyocin production was emphasized in this study, not only by the increased discrimination provided, but also by the high frequency of strains (74%) producing S-pyocins.

These improvements in technique and discriminatory power over the original cross-streaking method have resulted in greater potential of pyocin typing for most epidemiological purposes. One exception could be if only two or three strains are to be typed where the use of 13 plates might seem excessive.

It is interesting to note that the spotting method described here has recently been adapted successfully for the development of a bacteriocin typing scheme for P. cepacia (G. Harris & J.R.W. Govan, personal communication).

The method for pyocin detection described by Kageyama (1975), in which the spotting method was partially based, used a 'stab' inoculation technique for the application of producer strains. In

addition, pyocin production was induced by exposing the plates to UV following several hours incubation. In this study, the use of these procedures provided no advantages in terms of pyocin detection for typing purposes. Other differences between Kageyama's method and the method described in this thesis include the use of NA (by Kageyama) instead of TSA. No significant differences were noted between these two media, however, TSA was the medium of choice as it has been used successfully for pyocin typing using the cross-streaking method (Govan, 1978).

In this study, changing the incubation temperature was found to yield variable results, as different pyocins apparently have different optimum temperatures for production. This effect was not investigated systematically, however, the fact that certain S-pyocins were detected following incubation of the producer strains at 30°C, but not at 37°C, suggested that 30°C was a more suitable incubation temperature for typing purposes, thus confirming the findings of Gillies & Govan (1966).

It is interesting to speculate as to why the spotting method is more effective than the cross-streaking method for the pyocin typing of mucoid P. aeruginosa strains. Williams & Govan (1973) believed that the reason for non-typability of certain mucoid strains was that the alginate inhibited the diffusion of pyocins into the medium.

However, in this study, incubation of 30 mucoid P. aeruginosa isolates for 6h (i.e. under the conditions of the spotting method) resulted in detectable pyocin diffusion into the TSA medium. In the

case of some mucoid strains, no alginate could be detected (visually) following this short incubation, and indeed, mucoid strains have been shown to vary with respect to the stage of the growth cycle at which alginate is synthesized, i.e. in some strains alginate is synthesized constitutively (Mian et al, 1978), whereas in others it is a secondary metabolite with maximum rate of synthesis in the stationary phase (N. Piggott, PhD Thesis, Edinburgh University, 1979).

It is possible that the copious mucoid growth following the overnight incubation associated with the cross-streaking method, resulted in binding of certain pyocins which would then be removed along with the growth.

As expected, no problems were encountered in the pyocin typing of PA strains, as pyocin production does not appear to be influenced by surface changes of the cell.

Finally, the results obtained in this thesis from the typing of multiple P. aeruginosa isolates fromsingle and consecutive sputum specimens from five CF patients confirmed that in general, each patient was colonized (infected) with one particular strain on the basis of pyocin type, but that different isolates of that strain exhibited heterogeneous phenotypes with respect to alginate synthesis, antibiotic susceptibility etc. However, one of the six patients was found to harbour two different P. aeruginosa strains, and this may be relatively common, though the clinical significance of multiple colonization is unclear.

Early genetic studies in our own laboratory (Govan & Fyfe, 1978; Fyfe & Govan, 1980) had suggested that all strains of P. aeruginosa carry the genetic information necessary for the synthesis of alginate but that this information is normally repressed by some unknown mechanism. In addition, evidence for the chromosomal location of at least one gene associated with the repression of alginate synthesis in strain PAO had been obtained.

The research presented in Chapter VIII of this thesis was designed to obtain further information concerning the breakdown of the normal repression of alginate synthesis in both strain PAO and mucoid P. aeruginosa isolated from clinical specimens.

Firstly, a phenotypic screening of a large number of independent mucoid isolates, was performed with the view of identifying potentially different genotypes resulting in the derepression of alginate synthesis. Indeed, various different mucoid phenotypes within strain PAO were distinguished on the basis of their "medium-dependent" expression of alginate synthesis, and it was expected that variants expressing different mucoid phenotypes would result from mutations at different loci, as was the case with colanic acid synthesis in E. coli (Markovitz, 1977). Similar phenotypic differences among clinically isolated mucoid P. aeruginosa strains have been reported by Pugashetti et al (1982). In addition, these workers noted variation between strains with respect to alginate structure (e.g. ratios of D-mannuronosyl to L-guluronosyl moieties, and acetyl content of the polymers), and

stability of the mucoid phenotype on *in vitro* culture. However, there was no obvious correlation between these features. Similarly, Piggott (PhD Thesis, Edinburgh University, 1979) examined the composition of polymers obtained from various mucoid PAO derivatives (including PAO568 and PAO579), and noted that the mutants varied considerably, despite having been derived from the same parent strain.

However, phenotypic characterization provided little information regarding the number of genes associated with the repression of alginate synthesis. When ten independently isolated *muc* mutations in PAO were mapped by means of FP2-mediated conjugation and/or F116L-mediated transduction, nine were found to be closely linked to each other, near the catabolic marker *pru*, and the tenth, *muc-23* (which gave rise to medium-dependent expression of alginate synthesis similar to several of the other nine mutations), mapped at a different site.

Thus, it appears that the genetic basis of the variability of expression of alginate synthesis, at least in strain PAO is not readily explained on the basis of numerous loci associated with the repression of this characteristic. If indeed more than two loci are involved, they must be very closely linked.

Because of the close proximity of one of the two *muc* loci to the selectable catabolic marker, *pru*, it was possible to isolate R' plasmids carrying the *muc*$^+$ allele and various mutations at that locus.

Using these R' plasmids, preliminary studies on the expression of *muc* were performed. Merodiploid strains carrying the plasmid-determined *muc*$^+$ allele were invariably non-mucoid, suggesting that the *muc*$^+$ gene codes for a cytoplasmic regulatory product (as is the case with the *capR* gene product in *E. coli*) which is able to replace the non-functional mutant product.

Interestingly, merodiploid strains carrying a wild type chromosomal allele, and either *muc-2*, *muc-22* or *muc-45* as the plasmid determined marker, appear slightly mucoid on solid medium. This could be explained on the basis that these mutant genes code for a defective regulatory product which is synthesized in greater amounts when the gene is plasmid-determined (perhaps because the R' plasmids are present in multiple copies). However, it should be possible to isolate a *muc* mutant which produces no gene product, and such a mutation should be recessive to *muc*$^+$ in the merodiploid state, whether plasmid-determined or chromosomal.

Recently, Darzins & Chakrabarty (1984) have reported the cloning of genes controlling alginate biosynthesis from a mucoid CF isolate of *P. aeruginosa*. These workers obtained three recombinant plasmids (derived from broad host range vector pCA3) containing DNA inserts from the mucoid strain 8830. These inserts were considered to carry information associated with alginate biosynthesis as they were able to complement various Alg$^-$ mutations (of which they had identified four complementation groups). One of these recombinant plasmids, pAD1, was shown to induce alginate synthesis in PAO in a manner similar to the R' plasmids pJF54, pJF55 and pJF56. However,

the locus carried on pAD1, which complemented *alg-22*, has been mapped at approximately 19 mins on the PAO chromosome by virtue of the fact that pAD1 carries *argH*$^{+}$ on the same 7.5 kb DNA fragment. Hence the *alg-22* locus is unlikely to correspond to either of the *muc* loci mapped in the course of this thesis.

Goldberg & Ohman (1984) have also used cloning techniques to identify genes associated with alginate biosynthesis in a clinical mucoid strain fo *P. aeruginosa*. As with Darzins & Chakrabarty, these workers are primarily concerned with *alg* loci which are associated with the instability of alginate synthesis, and thus, not necessarily equivalent to the *muc* loci described here.

Following the isolation of the R' plasmids described in this thesis, it should be possible to physically characterize the *muc* gene and eventually identify its product. Shinomiya *et al* (1983) have used a similar approach for the characterization of the genetic determinant of pyocin R2 in *P. aeruginosa* strain PAO. Following the construction of R' plasmids carrying the chromosome segment containing the pyocin R2 gene cluster, these workers performed deletion mapping to further define the region and ultimately identified the pyocin R2 region as an insertion of 13 kb long, following heteroduplex analysis.

Recently, Darzins *et al* (1985) have succeeded in cloning the gene, *pmi*, coding for phosphomannose isomerase and have shown that mutations in this gene can lead to an Alg^{-} phenotype, thus emphasizing the essential role of this enzyme in the alginate biosynthetic pathway of their *P. aeruginosa* strain 8830.

An unexpected result from the R'studies reported in this thesis was the observation that pJF4 and similar R'*pru*$^+$*muc*$^+$ derivatives were KM^s, and that the KM^r phenotype returned when Pru$^+$ and Muc$^+$ were lost indicating that the presence of the chromosomal insert was responsible for inhibiting the expression of KM^r. The mechanism by which R' plasmids are derived from R68.45 was thought to be associated with the presence of the DNA sequence 1S21 which maps close to the KM^r determinant and contains two copies of a direct repeat, between which the chromosomal insert was thought to reside (Willetts *et al*, 1981). However, Moore *et al* (1983) have demonstrated that in the case of one R' plasmid derived by them, the chromosomal insert did not lie between the two copies of the direct repeat, but rather, one of the copies appeared to have been excised on the formation of the R'.

Furthermore, Beeching *et al* (1983), working with the closely related plasmid, R68.44, have demonstrated that the mobilization of a catabolic gene from the chromosome of *P. putida*, to form an R' plasmid, was not linked topographically with 1S21. Consequently, these workers have suggested that there may be mechanisms of R' formation which have nothing to do with the chromosome-mobilizing properties of the plasmid, i.e. the plasmid may simply act as a vector for transposable elements which leave the chromosome independently of any plasmid-determined mechanism. Indeed, transposable elements residing in the *P. aeruginosa* chromosome have been identified (Nash & Krishnapillai, 1982; Sinclair & Holloway, 1982).

With particular reference to alginate synthesis, Darzins & Chakrabarty (1984) have proposed that "the native genetic variation evident in P. aeruginosa suggests that a genetic mechanism other than point mutation may be responsible for the frequency of changes seen and the aspects of the phenotype involved". Examples of genetic rearrangements controlling microbial characteristics include (i) flagella phase variation in Salmonella typhimurium, where an invertible DNA sequence containing a promotor element controls the transcription of the H2 gene (Simon et al, 1980); (ii) pilus expression in Neisseria gonorrhoeae, where the conversion of the pilated to non-pilated state involves a chromosomal rearrangement (Meyer et al, 1982), and (iii) the antigenic variation in Trypanosome brucei which involves genomic rearrangements (Williams et al, 1979).

It would be interesting to determine whether the chromosomal region adjacent to pru, carrying the muc locus contains a transposon which could be associated with the expression of alginate synthesis. The fact that the region appears to be responsible for the derepression of alginate synthesis in nine out of ten mucoid derivatives of strain PAO and four out of five clinical mucoid isolates suggests that a mechanism other than point mutation is not unreasonable.

No light has so far been shed on the mechanism of control exerted by the muc gene product on the alginate biosynthetic pathway. Before this can be determined, the alginate biosynthetic pathway in P. aeruginosa must be completely elucidated and further genetic studies will need to be combined with biochemical ones.

The biosynthetic pathway leading to alginate in P. aeruginosa has been the subject of considerable interest and controversy since the mid 1970's. However, it is only in the last couple of years that a clear picture has begun to emerge.

The pathway shown in Figure 17 is a hypothesis based on the reports of several authors, the contribution of whom will be described. Firstly, the work of Banerjee et al (1983) provided evidence for the primary role of the Entner-Doudoroff pathway, and the importance of glyceraldehyde 3-phosphate in the synthesis of alginate from substrates such as glucose and gluconate. This followed studies of alginate synthesis in resting cell suspensions of mutants of a clinical mucoid strain of P. aeruginosa which were specifically defective in carbohydrate metabolism. Supporting evidence was provided by Lynn and Sokatch (1984) following studies of the pathway taken by ^{14}C from glucose substrates with carbon atoms specifically labelled with this isotope. Similarly, the role of fructose 1,6-bisphosphate aldolase was confirmed by Banerjee et al (1985) who demonstrated that a mutant deficient in this enzyme synthesized very little alginate from glucose and gluconate, but synthesized appreciable amounts from mannitol and fructose. Hence, glucose and gluconate must be converted to fructose 1,6-bisphosphate via the Entner-Doudoroff pathway and fructose 1,6-bisphosphate aldolase.

The section of the pathway leading from fructose 6-phosphate to alginic acid is based on that originally proposed for the marine brown alga Fucus gardneri (Lin & Hassid, 1966), and later Azotobacter vinelandii (Pindar & Bucke, 1975), and subsequently for P. aeruginosa (Piggott et al, 1981; Sutherland, 1982). However,

Fig 17. Proposed metabolic pathway for sugars leading to the biosynthetic pathway for alginate in P. aeruginosa [based on the studies of Banerjee et al (1983), Lessie & Phibbs (1984), Banerjee et al (1985), Darzins et al (1985), Piggott et al (1981) and Pugashetti et al (1983)].

The following abbreviations were used:
G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; KDGP, 2-keto 3-deoxy 6-phosphogluconate; Glyceraldehyde 3-P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; αGP, α-glycerophosphate; FBP, fructose 1,6-bisphosphate; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; Zwf, glucose 6-phosphate dehydrogenase; Edd, 6-phosphogluconate dehydrase; Eda, 2-keto 3-deoxy 6-phosphogluconate aldolase; Tpi, triose phosphate isomerase; Fba, fructose 1,6-bisphosphate aldolase; Fdp, fructose 1,6 diphosphatase; Pgi, phosphoglucose isomerase; pmi, phosphomannose isomerase; Pmm, phosphomannomutase.

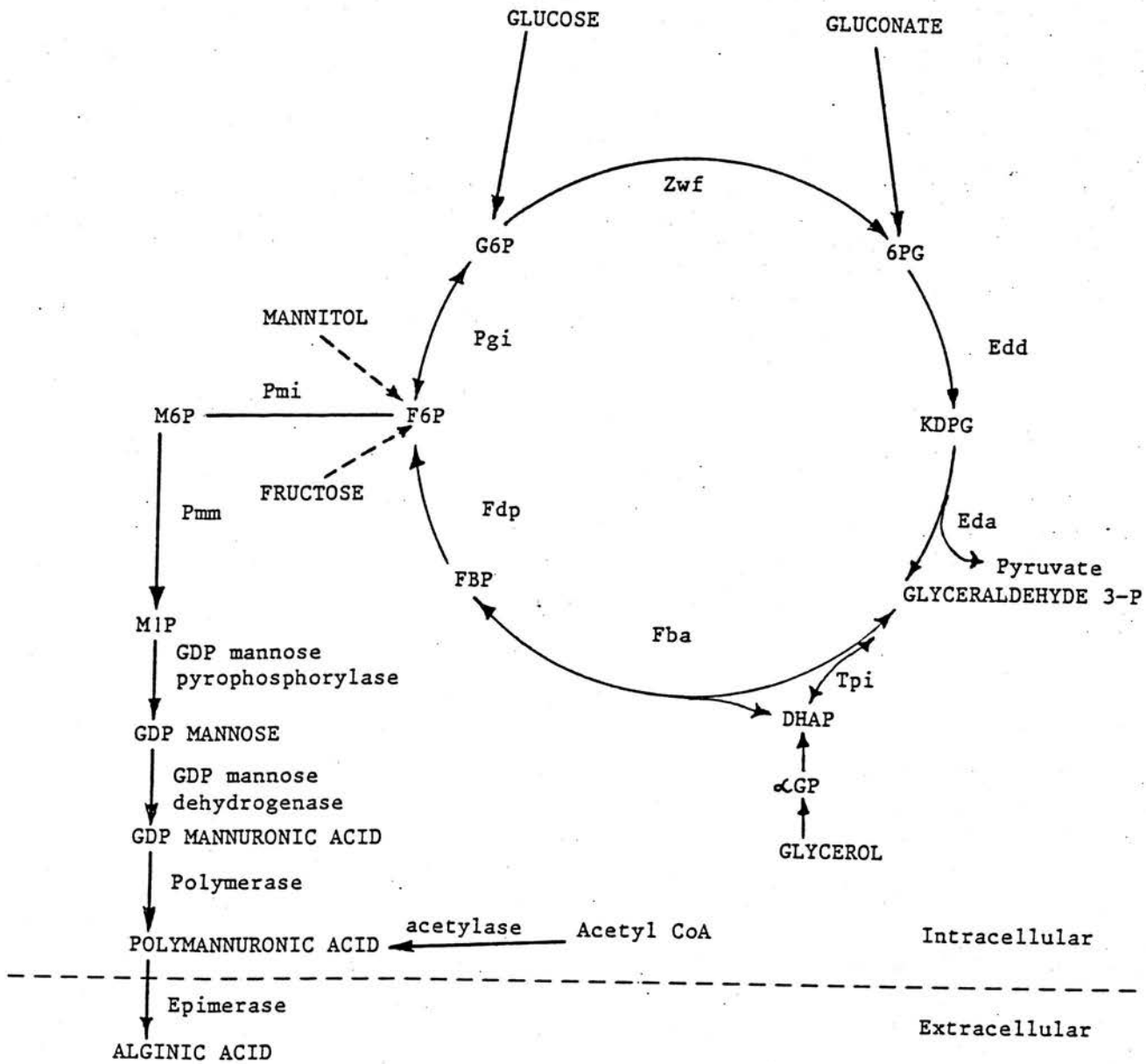

GLUCOSE
GLUCONATE
Zwf
G6P
6PG
MANNITOL
Pgi
Edd
Pmi
M6P
F6P
KDPG
FRUCTOSE
Pmm
Fdp
Eda
Pyruvate
FBP
GLYCERALDEHYDE 3-P
M1P
Fba
GDP mannose
pyrophosphorylase
Tpi
DHAP
GDP MANNOSE
GDP mannose
dehydrogenase
αGP
GDP MANNURONIC ACID
GLYCEROL
Polymerase
acetylase
Acetyl CoA
POLYMANNURONIC ACID
Intracellular
Epimerase
Extracellular
ALGINIC ACID

Figure 17

it is only recently that the role of fructose 6-phosphate as the precursor for alginate synthesis in *P. aeruginosa* has been confirmed. This was following the work of Darzins *et al* (1985) who successfully cloned the gene coding for the enzyme phosphomannose isomerase (*pmi*) from a strain of *E. coli* and transferred it to various *alg*$^-$ mutants of *P. aeruginosa*. The demonstration that the cloned gene complemented one particular class of *alg* mutant indicated that the mutation giving rise to the Alg$^-$ phenotype in that class was in the *P. aeruginosa pmi* gene, and that this enzyme is crucial for alginate biosynthesis in *P. aeruginosa*. Hence, the conversion of fructose 6-phosphate to mannose 6-phosphate appears to be the essential link between basic carbohydrate metabolism, common to all strains of *P. aeruginosa*, and the enzymes and intermediates specifically associated with alginate biosynthesis, only expressed by *muc* mutants.

In *E. coli* K12, phosphomannose isomerase is involved in both colanic acid synthesis and the utilization of mannose as a carbon source, indicating the bidirectional nature of the *E. coli* enzyme (Markovitz, 1977). However, *P. aeruginosa* is unable to utilize mannose as sole carbon source, either due to lack of an uptake mechanism for mannose and/or the unidirectional nature of the *P. aeruginosa* phosphomannose isomerase (i.e. the enzyme will only catalyse the reaction fructose 6-phosphate $\rightarrow$ mannose 6-phosphate and not the reverse). From the work of Darzins *et al* (1985), it would appear that the *P. aeruginosa* enzyme is indeed unidirectional, which is understandable on the basis of the lack of any DNA homology between the *E. coli* and *P. aeruginosa pmi* genes.

With respect to the enzymes catalyzing the steps leading from

mannose 6-phosphate to alginic acid, Piggott et al (1981) demonstrated elevated levels of GDP mannose pyrophosphorylase and GDP mannose dehydrogenase in mucoid P. aeruginosa PAO strains. Pugashetti et al (1983) have also shown elevated levels of the latter enzyme in a mucoid P. aeruginosa strain, compared with 18/20 non-mucoid revertants of that strain. The polymerization and acetylation steps probably take place at the level of the membrane and may involve lipid intermediates (Sutherland, 1982). Apparently, D-mannuronic acid is converted to L-guluronic acid by an extracellular epimerase, though it is still unclear whether this epimerization step takes place at the monomer or polymer stage.

Alginate synthesis by P. aeruginosa outside the environment of the CF lung is rare, suggesting that this property provides no selective advantage to the organism in its natural habitat. Consequently, a system of repression has evolved allowing the maintenance of the biosynthetic genes to provide the species with added adaptive potential. It would be interesting to establish whether one or both of the muc loci described in this thesis exert control over the alginate biosynthetic pathway by specifically repressing the transcription of the pmi gene, or whether the control mechanism involves the additional repression of other enzymes in the pathway. No doubt, this will soon be determined, however, it is sobering that after thirty years research, the mechanism by which capR regulates the colanic acid biosynthetic genes in E. coli (i.e. at the level of transcription) has only recently been elucidated (Trisler & Gottesman, 1984).

With respect to the evolutionary aspects of alginate biosynthesis in bacteria, it will be interesting to compare the

pathways in P. aeruginosa and A. vinelandii, in view of the close relatedness demonstrated between these two species on the basis of various characteristics e.g. close immunological relatedness of isofunctional proteins (Durham & Ornston, 1980), significant rRNA homology (De Vos, 1980), and a shared antigenic site on the major outer membrane protein H2 (Hancock et al, 1982). In view of the common ecological niche, i.e. the soil, shared by the genera Pseudomonas and Azotobacter, this is perhaps not surprising. However, unlike the pseudomonads, the majority of wild type A. vinelandii strains synthesize alginate, which in turn plays a crucial role in the cellular differentiation cycle of this organism leading to encystment (Lin & Sadoff, 1968; Eklund et al, 1966; Page & Sadoff, 1975).

The loss of intrinsic antibiotic resistance in P. aeruginosa

There is currently great interest in the structural basis of the intrinsic antibiotic resistance normally associated with P. aeruginosa. However, there have been few reports in the literature of genetic studies relating to this characteristic, other than plasmid-mediated antibiotic resistance (Jacoby, 1984).

It is generally believed that the intrinsic resistance of P. aeruginosa is associated with low permeability of the outer membrane of the bacterial cell envelope such that most antibiotics are excluded from their target sites on the cytoplasmic membrane or in the cytoplasm (Costerton & Cheng, 1975; Richmond & Curtis, 1974; Zimmerman, 1979; Yoshimura & Nikaido, 1982) Alternatively, some workers have argued that the penetration barrier may not be imposed by the outer membrane, but by a protein-glycoprotein layer adjacent

to the cytoplasmic membrane, or a periplasmic protein (Scudamore & Goldner, 1982).

Different approaches to the study of intrinsic antibiotic resistance in P. aeruginosa have been used by different workers, e.g. (i) the isolation of antibiotic hypersensitive, or resistant mutants of PAO (Mills & Holloway, 1976; Noguchi et al, 1980; Kropinski et al, 1978; Godfrey & Bryan, 1982; Nicas & Hancock, 1983); (ii) a comparison between the P. aeruginosa strain 799 and its antibiotic hypersensitive mutant 799/61 (Zimmerman, 1979; Zimmerman, 1980; Angus et al, 1982; Kropinski et al, 1982; Darveau & Hancock, 1983); or (iii) the study of clinical isolates differing in antibiotic susceptibility (Curtis et al, 1981; Irvin et al, 1981; Godfrey et al, 1981; Williams et al, 1984; Livermore, 1984).

The isolation of the related (on the basis of pyocin type) P. aeruginosa strains 492a and 492c from the same CF sputum specimen, differing markedly in antibiogram, provided an ideal starting point for the study of the structural and genetic basis of antibiotic sensitivity/resistance in association with this particular clinical situation.

May & Ingold (1972) had reported that 33% of their P. aeruginosa sputum isolates were more susceptible to carbenicillin than strains from other sources. These workers examined only single isolates from each specimen, hence it is not known whether these sputum specimens contained heterogeneous strains with respect to this characteristic. In a study of 33 sputum samples from 22 CF patients, Irvin et al (1981) reported that 12 of these patients harboured

P. aeruginosa strains sensitive to 1μg carbenicillin ml^{-1} and that from eight patients, isolates with normal carbenicillin susceptibility (i.e. MIC of 20 to 80μg carbenicillin ml^{-1}) were present simultaneously.

In this thesis, the MIC's of 18 antibiotics, in addition to carbenicillin, were determined for 492a and 492c. The latter strain was shown to be hypersensitive to methicillin, flucloxacillin, mecillinam, cefuroxime and naladixic acid, and was also more sensitive than 492a to chloramphenicol, tetracycline, trimethoprim and novobiocin. Because these antibiotics are associated with a range of different target sites and modes of action, this suggested that either 492c differed from 492a on the basis of several characteristics or a single difference in 492c accounted for its susceptibility to all these antibiotics, probably due to a change in outer membrane permeability. It is interesting that 492c is no more sensitive than 492a to the aminoglycosides, or the β-lactams benzyl penicillin, ampicillin, cephaloridine or cefoxitin. Presumably, even if 492c was more permeable to benzyl penicillin, ampicillin and cephaloridine, these β-lactams would be broken down before reaching their target sites by the inducible type 1d β-lactamase produced by the cells (Richmond, 1978). Cefoxitin, on the other hand, is resistant to the 1d β-lactamase, hence there can be no increased permeability to this antibiotic, despite the fact that 492c is hypersensitive to the closely related β-lactam, cefuroxime.

The possibility that 492c may have altered penicillin-binding proteins (PBP's) has not been investigated in this thesis. However, Curtis et al (1981) have examined the PBP's of two unrelated

clinical P. aeruginosa strains differing similarly, to 492a and 492c in their susceptibilities to β-lactam antibiotics, and found no significant differences. It was concluded that the strains differed with respect to their permeability barriers rather than PBP target affinity. Similarly, no differences in PBP's have been demonstrated for strains 799 and 799/61 (Zimmerman, 1980).

Livermore (1984) has recently reported a study of the PBP's and outer membrane permeability of clinical P. aeruginosa isolates resistant to carbenicillin (MIC >128μg ml^{-1}) independently of β-lactamase production. These strains are relatively common (Williams et al, 1984) and are also resistant to other β-lactams, naladixic acid, chloramphenicol and tetracycline, but not to aminoglycosides - a phenotype very similar to the nalB mutants in PAO (Rella & Haas, 1982). No evidence for altered target proteins was obtained for these strains and it was concluded that a reduction in outer membrane permeability was apparently responsible for the observed resistance.

The genetic studies described in Chapter IX used a combination of classical techniques to map two loci (blsA and tpsA) associated with antibiotic hypersensitivity in strain 492c by transferring to a PAO recipient. Similar interstrain crosses involving R68.45 have been used successfully in the mapping of xcp loci (Wretlind & Pavlovskis, 1983; Wretlind et al, 1983), and could also be used for the genetic analysis of other characteristics expressed by clinical strains, but not normally expressed by PAO (e.g. serum sensitivity).

When transferred into PA08, both the *blsA1* and *tpsA1* determinants contributed to the antibiotic hypersensitive phenotype, although *tpsA1* alone only caused increased sensitivity to naladixic acid, trimethoprim and novobiocin, and was also present in 492a.

The *tpsA* locus may not be associated with outer membrane permeability, but rather some intracellular function in view of the fact that the vegetative growth of F116L is apparently inhibited in strains carrying *tpsA1*.

In contrast, the *blsA1* gene was associated with hypersensitivity (or increased sensitivity) to β-lactams, chloramphenicol, trimethoprim, naladixic acid and novobiocin, strongly suggesting increased permeability as the likely mechanism. A phenotypically similar PAO mutant (PAO6511) was shown to carry a mutation in a locus indistinguishable from *blsA*.

The close promixity of *blsA* and *nalB* (which is associated with decreased permeability to the same antibiotics) on the PAO chromosome suggests that this region may contain a cluster of genes controlling the permeability of the *P. aeruginosa* outer membrane to these antibiotics, and possibly to other substances as well.

It would be very interesting to determine whether the carbenicillin resistant isolates described by Livermore (1984) and Williams *et al* (1984) carry mutations in the *nalB* locus, in view of their phenotypic similarity ot PAO *nalB* mutants. The clinical significance of this chromosomal region is apparent from the fact that two further antibiotic hypersensitive *P. aeruginosa* isolates

from CF patients carry blsA1-like mutations. Whether or not all clinical hypersensitive isolates are of a similar genotype remains to be determined. The hypothesis that a mechanism other than point mutation is operating to modify the outer membrane permeability of P. aeruginosa in certain selective environments would be an interesting one to test using the recombinant DNA techniques now available.

A genetic analysis of the antibiotic hypersensitivity in the mutant strain 799/61 was included in this thesis for two reasons. Firstly, to demonstrate that the approach used in mapping of blsA1 and tpsA1 could also be applied to the genetic analysis of 799/61, where previous approaches to this problem had been unsuccessful (Angus et al, 1982). Secondly, because of the importance of this strain in current studies on the structural basis of the intrinsic resistance of P. aeruginosa.

Strain 799/61 was isolated from 799 following multiple exposures to both EMS and N-methyl-N'-nitro-N-nitrosoguanidine and final screening on plates containing 0.5μg ml^{-1} cephalosporin C (Zimmerman, 1980). Hence it was not unexpected that at least two separate mutations were responsible for the extreme antibiotic sensitivity of this mutant. One of the mutations transferred from 799/61 to PAO222 (blsC4), was very closely linked to blsA and gave rise to a similar phenotype with respect to antibiotic hypersensitivity. The other mutation identified, blsB3, was cotransducible with pro-82 and gave rise to hypersensitivity to a wide range of antibiotics, from the relatively hydrophilic β-lactams to the hydrophobic agent, rifampicin. In view of the fact that the

double recombinant strain PAZ4 (blsB3, blsC4) remains resistant to cephaloridine (to which 799/61 is hypersensitive), it is likely that at least one additional mutation is responsible for the phenotype of 799/61, and this may be associated with the inducibility of the ld β-lactamase (799/61 is known to be defective in this function, Zimmerman, 1979).

The hypersensitive recombinant strains described in this thesis are now being used to examine the structural basis of antibiotic resistance and sensitivity in P. aeruginosa (B. Angus & R.E.W. Hancock, personal communication).

The low permeability of the P. aeruginosa outer membrane to hydrophilic solutes (including β-lactams) is largely due to the properties of the major outer membrane porin protein F (Hancock et al, 1979; Benz & Hancock, 1981; Yoshimura & Nikaido, 1982; Yoshimura et al, 1983). It is believed by most workers that this protein potentially forms relatively large water-filled channels or pores inthe outer membrane, but that only 0.2 - 1% of these are normally "open" (Angus et al, 1982; Yoshimura et al, 1983). It has been said that wild type P. aeruginosa behave like porin deficient mutants of E. coli (Hancock , 1980). The molecular mechanism underlying this remains unclear, though some workers believe that the association between protein F and the LPS is crucial in determining what proportionof the porin molecules form open, functional pores (Angus et al, 1982; Kropinski et al, 1982; Hancock, 1984). This hypothesis is largely based on the finding that 799/61 has an altered LPS (Darveau & Hancock, 1983) though it has not been

shown conclusively that this is associated with increased outer membrane permeability.

Genetic and functional studies of the P. aeruginosa outer membrane are at present in their early stages. However, certain approaches applied to similar problems in E. coli may provide clues as to the direction to follow in the future, even though the answers may prove to be different.

In E. coli K12, there are two-major outer membrane porin proteins, OmpF and OmpC (reviewed by Nikaido & Vaara, 1985) which are coded for by the chromosomal genes ompF and ompC respectively (Reeves, 1979). It has been shown that the OmpF and OmpC proteins facilitate the penetration of β-lactams through the outer membrane (Jaffe et al, 1982; Nikaido et al, 1983), and mutants lacking these proteins can be obtained by selecting for resistance to cefoxitin (Jaffe et al, 1982). In contrast, a hyperpermeable E. coli mutant, DC2 (Richmond et al, 1976) appears to have no alteration in outer membrane proteins, and it has been suggested that the mutation creates a bypass of the porin system (Jaffe et al, 1982). Another class of E. coli mutants hypersensitive to both hydrophobic and hydrophilic agents have mutations at the envA locus. Such mutants have no obvious outer membrane protein or LPS defects but may have altered levels of phospholipids in their outer membranes (Boman et al, 1974; Grundstrom, 1980).

A combination of recombinant DNA and biochemical analysis has been used very neatly to determine the gene product associated with tolC locus in E. coli K12 (Morona et al, 1983). tolC mutants become

tolerant to colicin El, have an altered bacteriophage sensitivity pattern, lack the OmpF protein and become hypersensitive to detergents, dyes and certain antibiotics. Polyacrylamide gel electrophoresis (PAGE) of a TolC strain did not at first reveal the absence of any protein band which might have been the tolC gene product. However, cloning of the tolC gene allowed the amplification and identification of the product as an outer membrane protein required at a post-transcriptional step inthe expression of OmpF.

To return to antibiotic hypersensitivity in association with a clinical situation, it is interesting to consider the case of N. gonorrhoeae, where 15% of clinical isolates in one study were env mutants (Eisenstein & Sparling, 1978). Sarubbi et al (1975) have shown that there are three env loci on the N. gonorrhoeae chromosome, two of which are very closely linked, and one genetically separate. To explain this observation, it has been proposed that wild type enteric bacteria possessing outer membranes of low permeability to hydrophobic molecules may have a selective advantage when exposed to intestinal bile salts and long chain fatty acids. However, for a mucosal surface pathogen, such as the gonococcus (and possibly P. aeruginosa in the respiratory tract), it may be advantageous to be more flexible with respect to the structure of the outer membrane (Lysko & Morse, 1981). Whether or not the structural basis of antibiotic hypersensitivity is similar in N. gonorrhoeae and P. aeruginosa remains to be seen, but it is interesting to note that the porins in the outer membrane of N. gonorrhoeae are large like those of P. aeruginosa, excluding only

those saccharides of MW >6,000 (Douglas et al, 1981, cited by Hancock, 1984).

Clinical implications

A noted CF researcher has concluded that "an understanding of the basic biology of Pseudomonas in the CF respiratory tract is crucial to the achievement of infection control" (Thomassen et al, 1977).

The research presented in this thesis was not primarily designed to examine the therapeutic problems of pulmonary infection due to P. aeruginosa, but rather to approach a better understanding of the basic biology of pulmonary isolates of P. aeruginosa.

It had long been assumed that classic, non-mucoid P. aeruginosa in the CF respiratory tract were of no clinical significance, and a careful, statistically validated study has recently confirmed this view (Henry et al, 1982). However, transition to the mucoid form is correlated with deterioration in lung function, and mucoid P. aeruginosa are thus considered pathogens.

The clinical relevance of the other characteristics associated with P. aeruginosa pulmonary isolates from chronically ill patients (i.e. antibiotic hypersensitivity, serum sensitivity, loss of the specific O-antigen and PA) has so far received less attention. Penketh et al (1983b) attempted to correlate the clinical condition of a group of CF patients with the characteristics expressed by their P. aeruginosa isolates (i.e. serum sensitivity, loss of O-antigen and PA). They observed that patients carrying strains which

expressed some, but not all of these characteristics, i.e. "intermediate" strains, were poorer clinically than those patients carrying classic strains or strains expressing all characteristics. This could be interpreted to mean that the process of surface adaptation of the organisms within the CF lung upsets some kind of equilibrium between bacteria and the host defences, which results in greater damage to lung tissue, mediated by either the host or the bacteria or both. Once the organisms have adapted, the equilibrium is restored.

It is is not clear, at present, in what order these characteristics are expressed by pulmomary isolates of P. aeruginosa in association with chronic illness, and if they emerge in the same order in each case. The genetic basis of alginate synthesis and antibiotic hypersensitivity have been shown to be different and distinct from serum sensitivity and PA. However, it is possible that serum sensitivity and PA could result from a single genetic change.

It would be reasonable to propose that initial colonization of the upper respiratory tract or tracheobronchial mucin of the CF patient (or bronchiectic) by a classic, non-mucoid strain of P. aeruginosa would eventually give rise to a large population of cells at these sites. Mucoid mutants arising from this population would then multiply to form gel-enclosed microcolonies, better able to resist phagocytosis by alveolar macrophages and thus capable of colonizing the smaller airways. Once established in the protected microenvironment of a blocked airway, surrounded by an alginate gel,

growth under such conditions may favour a more permeable outer membrane, providing better access to the limited nutrients. Thus, the antibiotic hypersensitive strains would be selected for. Presuming that this characteristic is actually expressed *in vivo*, it would appear that the aggressive intravenous antibiotic therapy often administered to these patients results in insufficient levels of antibiotic to kill even hypersensitive organisms, at the site of infection. This could be the result of poor access to the lung, or alternatively binding and breakdown of the antibiotic by the abnormal secretions, and alginate.

It is possible that serum sensitivity and PA emerge prior to alginate synthesis and antibiotic hypersensitivity, as these characteristics may be associated with evasion of antibody-mediated killing.

P. aeruginosa products, such as exotoxin A and elastase, may or may not directly cause damage to lung tissue during chronic infection. However, the presence of large numbers of bacteria in the lungs, particularly of a CF patient, is associated with a poor prognosis. The process by which the organisms become established in large numbers in the lungs is long and involves a number of genetic changes which are then selected for in a particular environment. It is important to understand this process of adaptation and the potential of *P. aeruginosa* in this situation. There is no clear-cut distinction between *P. aeruginosa* the harmless colonizer, and *P. aeruginosa* the pathogen - one emerges from the other, and to treat the patient only when obvious signs of deterioration become evident is to "close the door after the horse has bolted".

It is important to determine the factors responsible for the intial colonization of the respiratory tract by P. aeruginosa and to apply the old idea that "prevention is better than cure".

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Revised Pyocin Typing Method for *Pseudomonas aeruginosa*

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In the Gillies and Govan method of pyocin typing for *Pseudomonas aeruginosa* a cross-streaking technique was used, and 105 main types and 25 subtypes were identified by the patterns of inhibition observed on 13 indicator strains. Disadvantages of the technique included the need to remove test strain growth before application of the indicator strains, the 48-h period needed to obtain a result, and the inability to reliably type mucoid *P. aeruginosa*. Recent studies have enabled us to overcome these disadvantages and significantly improve the speed and application of pyocin typing. Our revised technique utilizes the same 13 indicator strains which are already used internationally. Test strains were rapidly applied to the surface of agar plates with a multiple inoculator. After incubation for 6 h and exposure to chloroform, the indicator strains were applied in agar overlays without prior removal of the test strain growth. After 18 h of incubation, the pyocin type was recognized by inhibition of particular indicator strains. Additionally, the activity of particulate (R and F) and nonparticulate (S) pyocins could be distinguished on the basis of inhibition zone size, which thus allowed further discrimination. The revised technique allows typing within 24 h, increases the number of identifiable types, and can be used to type mucoid strains.

In the last three decades, *Pseudomonas aeruginosa* has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients (12). At particular risk are patients who have suffered major trauma or burns and are exposed to intensive care units (16). Also at risk are normal individuals exposed to a compromising occupational or recreational environment, e.g., a deep-sea diving bell, in which outbreaks of acute and painful otitis externa due to *P. aeruginosa* have been reported (1), or the jacuzzi, in which *P. aeruginosa* has been implicated in the irritating skin rash known as "hot tub" or jacuzzi syndrome (17).

The increased importance of *P. aeruginosa* as an opportunistic pathogen, together with its well-recognized and characteristic ubiquitous nature, gives rise to many instances in which reliable and discriminating typing or "fingerprinting" of strains is required to investigate outbreaks of nosocomial infection and to aid effective infection control.

Several biological criteria have been assessed for typing *P. aeruginosa*, including pigmentation, antibiograms, and phage sensitivity. However, the two most reliable and generally accepted methods are serotyping and pyocin (aeruginocin) typing (2, 14). A number of pyocin typing methods have been described, but in independent comparative experience and reviews (2, 11) it has been suggested that the most suitable method is that developed in our laboratory (4, 7, 8).

In our pyocin typing technique (7), a cross-streaking method is used which can identify 105 main types on the basis of pyocin production by test strains and the recognition of different inhibition patterns observed against eight indicators, labeled 1 through 8, and further subdivision into 25 possible subtypes with five additional indicator strains, labeled A through E. Thus, the discriminatory potential of the method is good and superior to serotyping. However, in epidemiological studies, as with serotyping, the majority of strains fall into a limited number of types or subtypes. Thus, further discrimination would be an advantage. Other disadvantages of this method are (i) the 48 h required to obtain a result; (ii) the need to remove the producer strain growth before application of the indicator strains, which is a messy and time-consuming procedure; (iii) the inability to reliably type mucoid strains of *P. aeruginosa*, which have become a serious problem in respiratory infections in patients with cystic fibrosis; we have previously described a modified pyocin typing method for mucoid *P. aeruginosa* (18), but the method involves preparation of pyocin-containing extracts from aerated broth cultures, and although simple to perform, it is time consuming and labor intensive; and (iv) the fact that the technique, as first described in 1966 (4), did not distinguish between the different classes of pyocin produced by *P. aeruginosa*, i.e., the particulate R- and F-pyocins (5, 6) and the diffusible S-pyocins (9). It has been suggested (7) that the ability to distinguish between particulate and nonparticulate pyocins on the basis of inhibition zone size would provide additional valuable strain discrimination in pyocin typing.

Our continued studies on the production and detection of individual pyocins, including their production by mucoid *P. aeruginosa*, have led to the reduction or elimination of these disadvantages and, thus, to significant improvements in the speed, sensitivity, and application of pyocin typing for epidemiological purposes.

This paper follows a preliminary report (J. A. M. Fyfe and J. R. W. Govan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C35, p. 317) and describes the development and evaluation of a revised technique for pyocin typing. In the revised technique, the use of the original 13 indicator strains and the inhibition patterns previously described (7) are retained. The main practical modifications to the previously described cross-streaking technique (7) are that the test strains are applied by a spotting method and the indicator strains are incorporated in agar overlays without prior removal of the test strain growth.

MATERIALS AND METHODS

Bacterial strains. Fifty pyocinogenic strains of *P. aeruginosa* were employed initially to compare the pyocin typing results obtained by the standard cross-streaking method and a modified spotting method. Thereafter, an additional 500 clinical isolates of *P. aeruginosa*, including mucoid strains, were used to evaluate the use of the spotting method.

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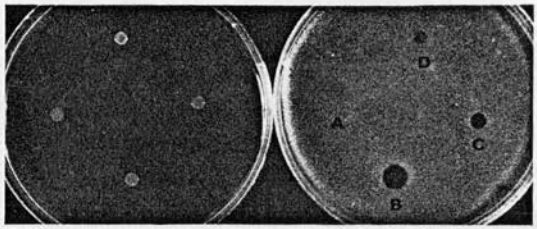

FIG. 1. Examination for pyocin activity against indicator strain 8 in four strains of *P. aeruginosa* by the spotting method. The left plate shows the test strains A, B, C, and D after 6 h of incubation at 30°C and before addition of the indicator strain. The right plate shows the inhibition zones produced by those strains after addition of the indicator strain in an agar overlay and subsequent incubation for 18 h at 37°C. Strain A shows no pyocin activity (−); strain B shows S-pyocin activity (+) characterized by an inhibition zone extending beyond the area of original growth; strain C shows a restricted inhibition zone characteristic of R- and F-pyocins (+); strain D shows a mottled inhibition zone typical of phage activity (-Ø).

Pyocin typing by the cross-streaking method. Pyocin typing by the cross-streaking method was carried out as previously described (7). As suggested in this review, the recognition of S-pyocin activity was incorporated into the typing scheme. Thus, a test strain was allotted to a pyocin type not only on the basis of the pattern of inhibition observed against the 13 standard indicator strains, but it was further characterized by noting the presence of classic S-pyocin activity which causes a zone of inhibition that extends beyond the original growth area of the producer strain. Hence, for example, a strain of pyocin type 1/a producing S-pyocin against indicator strains 7 and B is designated type 1/a ($S_{7,B}$).

Pyocin typing by the spotting method. Strains of *P. aeruginosa* to be typed were streaked for single colonies onto nutrient agar (Columbia agar base; Oxoid Ltd., Basingstoke, London, England) and incubated at 37°C overnight. The single colonies that arose from each test strain were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1 ml of sterile physiological saline (absorbance at 550 nm, ≈0.5).

A multipoint inoculator (model A400; Denley Instruments Ltd., Sussex, England), incorporating 21 stainless steel pins (one being a marker pin; diameter of each pin, 2 mm; pins were set 16 mm apart), was used to dispense 1-μl volumes of the bacterial suspensions onto a set of 13 plates (diameter, 90 mm) each containing 10 ml of tryptone soy agar (Oxoid). In this way, 20 test strains could be typed simultaneously against each indicator strain. After the spots dried, usually within a few minutes, the plates were incubated at 30°C for 6 h. Filter paper disks (5 cm; Whatman, Inc., England) were impregnated with chloroform, and the plates were placed over the disks for 15 min to allow the chloroform vapor to kill the bacteria. The plates were then exposed to air for an additional 15 min to eliminate residual chloroform vapor. Cultures of the indicator strains, grown without agitation in nutrient broth (Oxoid no. 2) for 4 h at 37°C to a population size of approximately 10^7 organisms per ml, were applied to the plates by adding 0.1 ml of each bacterial indicator culture to 2.5 ml of molten, semisolid agar (1% peptone; Difco Laboratories, Detroit, Mich., in 0.5% agar; Oxoid L 11) held at 45°C and poured as overlays (NB, a separate indicator strain, was applied to each plate). When the overlays had set, the plates were incubated for 18 h at 37°C, and the pyocin types were determined, as with the cross-streaking method, on the basis of the indicator strains inhibited. The size of the inhibition zones was also taken into account for the purpose of more detailed strain comparison (Fig. 1) and determination of S-pyocin activity incorporated into the typing results as in the cross-streaking method.

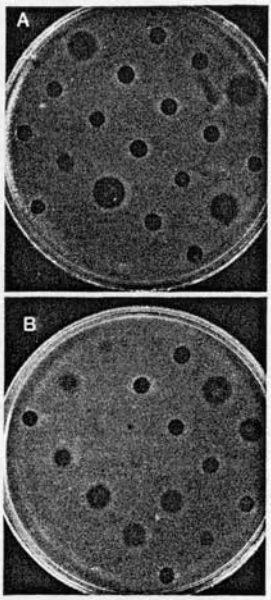

FIG. 2. Typical routine typing plates obtained by the spotting method. (A) Indicator strain 5; (B) indicator strain B.

RESULTS

Detection of pyocin activity by the spotting method. Incubation of the test strains at 30°C on tryptone soy agar for 6 h resulted in detectable pyocin production on the basis of clear zones of inhibition of an indicator strain (Fig. 1). S-type pyocin production could be distinguished from R- and F-types on the basis of zone size. The area of growth after inoculation with a multipoint inoculator had a diameter of 5 mm. Inhibition zones due to R- or F-type pyocins ranged from 5 to 7 mm in diameter and had a sharp edge. S-type pyocins produced zones of 9 to 12 mm in diameter, and these often had a diffuse edge. Phage activity could generally be distinguished from pyocin activity, as the former gave rise to a mottled inhibition zone. Two typing plates on which the same 20 test strains were overlaid with indicator strain 5 (Fig. 2a) and indicator strain B (Fig. 2b) are shown. Comparison of test strains grown on the same set of plates is easier and more meaningful than comparison of those grown on individual plates, as in the cross-streaking method.

Comparison between spotting and cross-streaking methods. For comparative purposes, 50 strains of *P. aeruginosa* were typed by both methods. Twenty-seven strains gave identical results, including the detection of S-type pyocin activity. Nine strains gave the same pyocin type, although the cross-streaking method failed to detect S-pyocin activity against one or more indicators. Fourteen strains gave a different pyocin type by the two methods. In 12 strains, pyocin activity was detected by the spotting method, which failed to

be detected by the cross-streaking method, and in the remaining 2 strains pyocin activity was detected by the cross-streaking but not the spotting method.

Value of S-pyocin production as a further epidemiological discriminator. An additional 500 clinical isolates of *P. aeruginosa* were pyocin typed by the spotting method. The majority of these strains (99%) were typable, and 74% produced S-pyocin activity against 1 or more of the 13 indicator strains. All indicators showed some sensitivity to S-pyocins, and 63 different "S-type patterns" were distinguished. The most common of these were S_5 (11.3% of those strains with S-pyocin activity), $S_{5,B}$ (9.7%), S_7 (8.9%), and $S_{7,B}$ (8.6%).

As an example of the improved discrimination provided by recognition of S-pyocin activity, 34 isolates of *P. aeruginosa*, belonging to the common pyocin type, 1/b, could be divided into 10 distinct groups on the basis of their S-pattern.

Typing of mucoid strains by the spotting method. Thirty mucoid strains of *P. aeruginosa* isolated from the sputa of six patients with cystic fibrosis were pyocin typed by the spotting method. These included multiple isolates from individual sputa. All 30 strains gave clear typing patterns, with 13 strains producing S-pyocins. Multiple isolates from five of the patients were shown to be of the same type (different types for each patient), whereas the sixth patient harbored strains of two distinct types, 29/f(S_5) and 13/k.

DISCUSSION

The revised spotting method of pyocin typing described in this report has advantages over the cross-streaking method previously described (7). (i) The time required to obtain a typing result is reduced from 48 to 24 h. (ii) Up to 20 isolates can be directly compared on the same set of typing plates. This is ideal for comparative typing of multiple colonies from a single specimen to investigate mixed-type infection or to compare isolates from a single epidemic outbreak. (iii) Inhibitory activity due to S-pyocins can be more readily distinguished from R- and F-pyocin activity than when the cross-streaking method is used, thus providing greater discrimination in epidemiological studies. In our study, the epidemiological value of including detection of S-pyocin activity has been emphasized not only by the increased discrimination which it provides but also by the high frequency of S-pyocin production (74%) observed in the 500 test strains examined. This incidence is higher than that reported in a study carried out in India (38%); in the latter survey, however, only 29 strains were examined, and the methods for pyocin production and detection included different cultural conditions and indicator strains (15). (iv) There is no requirement to remove producer strain growth before application of the indicator strains; hence, the method is less tedious and time consuming. Similarly, the application of the indicator strains in agar overlays rather than as cross-streaks is more efficient. (v) Finally, the spotting method is more suitable for typing mucoid *P. aeruginosa*.

An earlier spotting method for pyocin studies described by Kageyama, in which different cultural conditions were used (10), included induction of pyocin production by exposure of the producer strain to UV light. We made a comparative study of six standard reference strains with a range of UV doses and found that under the conditions of the revised typing technique described in this report, no significant advantage was gained for typing purposes by inclusion of an induction stage (unpublished data). In addition, for typing purposes, an induction stage to enhance pyocin production in apparently apyocinogenic strains and thus to reduce the number of untypable strains was unnecessary due to the very low incidence of such strains (1%) found in our survey.

In a wider context, the question remains as to which is the most suitable typing system for epidemiological studies of *P. aeruginosa*, and realistically, is any one system adequate? Despite the improvements in pyocin typing described in this paper, the method still does not match the rapidity of the other most-suitable typing method, serotyping.

P. aeruginosa is serologically heterogeneous, and identification of group-specific heat-stable lipopolysaccharide antigens by agglutination forms the basis of O-serotyping procedures. Several systems have been described and their use reviewed (2, 11, 14). O-serogroup sera are available commercially, but they are expensive and the most widely used system (Difco) requires a set of antigen suspensions for characterizing the sera. In addition, the sera can only be purchased as a complete set of 17 sera.

A major disadvantage of O-serotyping is that the discriminatory power is only fair (3); further discrimination can be provided by detection of H-antigens, but the procedures for H-typing are beyond the scope of many laboratories (14). The typability of *P. aeruginosa* by O-serological typing is usually over 90%, but serotyping is often unsatisfactory for mucoid *P. aeruginosa* in which O-antigens may be masked, typing of colonial dissociants in which serological changes occur within a single culture, and typing polyagglutinable *P. aeruginosa*. The latter, together with mucoid *P. aeruginosa*, forms less than 5% of clinical isolates but is frequently observed in patients with cystic fibrosis (13).

By our use of the revised pyocin typing technique described in this report in combination with O-serotyping it is concluded that neither system provides all the requirements of the ideal typing system for *P. aeruginosa* (unpublished data). We suggest that both systems offer significant contributions to epidemiological studies. O-serotyping provides a rapid indication of antigenic differences when these occur. In an epidemic situation, however, the value of serotyping is limited unless the strains isolated belong to unusual serotypes. Pyocin typing, as described in this paper, requires a period of 24 h to achieve a result but provides adequate discrimination on which to base more confident epidemiological judgment. We suggest that this revised technique provides an improved method for both epidemiological studies of *P. aeruginosa* and for basic studies on the wide range of pyocin activity which can be found in this species.

ACKNOWLEDGMENTS

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Heterogeneity and Reduction in Pulmonary Clearance of Mucoid *Pseudomonas aeruginosa*

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Mucoid, alginate-producing *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis were studied to determine the extent of heterogeneity of the isolates within individual sputa. Considerable heterogeneity involving cultural requirements for mucoid colonial growth was observed, and sensitivity to the β-lactam antibiotic carbenicillin was also variable. For determining if the presence of alginate increased pulmonary survival of the bacteria, groups of rats were infected by transtracheal instillations of equivalent numbers of mucoid or nonmucoid *P. aeruginosa*, and survival was measured as the percentage of inoculum colony-forming units cultured from lung homogenates. Increased pulmonary survival of mucoid *P. aeruginosa* was observed in animals killed 3 hr or 6 hr after infection with unwashed bacteria. No difference in survival between mucoid and nonmucoid cells was observed when bacteria were washed prior to instillation. It was concluded that a single mucoid colony isolated from a sputum does not fully represent the population of mucoid *P. aeruginosa* within the patient and that pulmonary killing of mucoid *P. aeruginosa* can be less efficient than that for nonmucoid strains.

In the late 1960s the pioneering research of Doggett and Harrison [1] revealed the association of mucoid *Pseudomonas aeruginosa* and chronic, severe pulmonary infection in patients with cystic fibrosis. Today, *P. aeruginosa* has become the most troublesome respiratory pathogen of patients with cystic fibrosis [2, 3]. Although research in the last decade has greatly increased our basic knowledge of the properties of this organism, the natural history of pseudomonas infection in patients with cystic fibrosis is not fully understood.

Clinical and bacteriologic studies of patients with cystic fibrosis have indicated that initial infection occurs with a classic, nonmucoid strain of *P. aeruginosa*. Subsequently, a mucoid form emerges and becomes predominant with concomitant pulmonary deterioration and a poor prognosis for the patient [1–4]. Nonmucoid and mucoid forms isolated from the same patient usually belong to the same pyocin type [5] and serotype [6].

The exopolysaccharides produced by mucoid *P. aeruginosa* isolated from 10 patients with cystic fibrosis were shown to be similar and to consist of an acetylated alginate [7]. In contrast to pseudomonas slime [8], purified pseudomonas alginate is nontoxic when injected ip into mice (authors' unpublished results). Several in vitro studies have indicated that the alginate may be antiphagocytic [9, 10]. It would seem probable, therefore, that the function of the alginate is protective in vivo rather than invasive.

It has been reported [3] that the continuous use of antibiotics seems to contribute to the emergence of mucoid strains. Some mucoid variants have been reported to be slightly more resistant to β-lactam antibiotics and aminoglycosides than are related nonmucoid strains, and the isolation of mucoid, alginate-producing mutants of *P. aeruginosa* [11] and other *Pseudomonas* species [12] with use of a technique based on enhanced resistance of these mutants to carbenicillin has been described. It must be emphasized that generalizations cannot be made about the resistance of mucoid strains or their nonmucoid revertants. Some revertants retain the enhanced resistance to carbenicillin of the mucoid parent strain [13]; more striking, perhaps, is that very sensitive strains of both mucoid and nonmucoid forms of

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P. aeruginosa frequently are isolated from sputa [14].

This paper reports on the heterogeneity of mucoid *P. aeruginosa* isolated in vitro and from patients with cystic fibrosis and on the use of transtracheal inoculation in rats to compare the pulmonary clearance of isogenic mucoid and nonmucoid strains. The significance of the results with respect to present knowledge of pseudomonas infection in patients with cystic fibrosis is then discussed.

Materials and Methods

Pseudomonas aeruginosa strains. Mucoid strains of *P. aeruginosa* were isolated from sputa obtained from patients with cystic fibrosis attending the cystic fibrosis clinic at the Royal Hospital for Sick Children, Edinburgh. The mucoid mutant strain PAO 579 [15] was isolated from a culture of strain PAO 381 with use of a carbenicillin selection technique [11].

Heterogeneity of mucoid P. aeruginosa in sputa of patients with cystic fibrosis. Sputa of patients were treated with sputolysin (Calbiochem-Behring, Bishop's Stortford, England), diluted in sterile physiologic saline, and plated on Pseudomonas isolation agar (PIA; Difco, Detroit, Mich.). After incubation for 24 hr at 37 C, 100 mucoid cfu resembling Phillips colonial type 5 [16] were subcultured onto nutrient agar (NA; Columbia agar base, Oxoid, London), blood agar (BA; Columbia agar supplemented with 5% sterile human blood), minimal agar (MA, [17]), deoxycholate citrate agar (DCA; Oxoid CM227), and diagnostic sensitivity test agar (Oxoid) containing 5 μg of carbenicillin/ml (Beecham Research Laboratories, Brentford, England) and examined for mucoid growth after 24 hr at 37 C.

Pseudomonas alginate. The exopolysaccharide produced by mucoid strains grown on PIA was extracted, purified to an asbestos-like powder, and identified as alginate as previously described [11]. Gelatinization of the exopolysaccharide was examined by preparing a 1% aqueous solution from dried alginate and adding 3 m*M* $CaCl_2$.

Animal studies. Pathogen-free male Sprague-Dawley rats (200 g) were obtained from OLAC 1976 (Bicester, England). The technique used for transtracheal inoculation of bacteria into a distal bronchus was that previously described [18]. Cultures of strains PAO 381 and PAO 579 were grown in a chemically defined medium [19] with 1% sodium gluconate as the carbon source and were incubated in an orbital incubator (Gallenkamp, London, England) at 140 rpm at 37 C for 18 hr to stationary phase. Strain PAO 579, but not strain PAO 381, readily produces extracellular alginate under these in vitro conditions, but no clearly defined bacterial capsule is observed in preparations stained with india-ink or ruthenium red. The bacterial cultures were diluted 1:100 in PBS (pH 7.0); the 0.1-m inoculum contained $\sim 10^6$ cfu. Washed bacteria were prepared by two cycles of centrifugation at 3,000 *g* and resuspension of bacteria in PBS to yield 10^7 cfu/ml.

Bacterial quantitation. Animals were killed with ip pentobarbital, and both lungs and the heart were removed aseptically and washed with sterile PBS. The lungs were then excised and placed in 10 ml of sterile PBS. The tissues were homogenized in a Waring blender. Serial dilutions of the homogenate in PBS were plated on DSTA, and cfu were counted after overnight incubation at 37 C.

Pyocin typing. The pyocin-typing technique used was that described by Williams and Govan [5], with the incorporation of the revised scheme of Govan [20].

Results

Heterogeneity of mucoid P. aeruginosa. The heterogeneity of mucoid *P. aeruginosa* was discovered by the isolation of variants that produced alginate only when grown on certain media and, in addition, by the identification of mucoid variants that were sensitive to 5 μg of carbenicillin/ml.

Table 1 summarizes the characteristics of four groups of "medium-dependent variants" of *P. aeruginosa* isolated from patients with cystic fibrosis. These variants were recognized by their ability to produce mucoid growth resembling the Phillips colonial type 5 [16] after incubation at 37 C for 24 hr. Mucoid mutants belonging to groups 1–4 were successfully isolated in vitro from the nonmucoid strain PAO 381 as single-step mutations with use of the carbenicillin selection technique. The exopolysaccharide from each mutant gelled rapidly in the presence of Ca^{++} (figure 1) and was identified as an acetylated alginate-like polymer of mannuronic and guluronic acid.

Table 2 summarizes the incidence of each mucoid group and of the strains of *P. aeruginosa*

Table 1. Characterization of medium-dependent mucoid variants of *Pseudomonas aeruginosa* isolated in vitro and from patients with cystic fibrosis.

Variant group	Type of growth in indicated medium*			
	PIA	NA/BA	DCA	MA
1	+	+	+	+
2	+	+	+	−
3	+	+	−	−
4	+	−	−	−

* + = mucoid growth resembling Phillips colonial type 5 after incubation at 37 C for 24 hr; − = nonmucoid growth; PIA = pseudomonas isolation agar; NA = nutrient agar; BA = blood agar; DCA = desoxycholate citrate agar; and MA = minimal agar [17]. See Materials and Methods for further information about the media.

Table 2. Heterogeneity of mucoid *Pseudomonas aeruginosa* isolates from 100 sputum samples obtained from 17 patients with cystic fibrosis.

Group of mucoid isolate*	Percentage of sputum samples harboring group	Percentage of patients
1	89	100
2	24	65
3	10	35
4	11	29
Carbenicillin sensitive†	36	65

NOTE. Data are based on the analysis of 100 mucoid colonies from each sputum sample.

* See table 1 for characterization of mucoid groups.

† MICs of carbenicillin for isolates were <5 μg/ml.

sensitive to 5 μg of carbenicillin/ml among all strains isolated from 17 patients with cystic fibrosis who were chronically infected with this organism. Thirty-five sputum samples obtained from three patients presented a homogenous population of group 1 mucoid strains of *P. aeruginosa*. Indeed, variants belonging to group 1 were by far the most common; they were isolated from all 17 patients and from 89% of the individual sputa. Variants belonging to group 2 were isolated from 65% of patients; group 3 variants, from 35%; and group 4, from 29%. Strains of *P. aeruginosa* sensitive to 5 μg of carbenicillin/ml were isolated from 65% of the patients. This characteristic was independent of the mucoid group. Although most patients harbored mucoid strains from more than one group simultaneously, no clear transition was observed from one group to another. Group 1 variants usually appeared first in the course of infection and accounted for the majority of *P. aeruginosa* isolated from an individual specimen or patient. However, mucoid variants sensitive to <5 μg of carbenicillin/ml also were found frequently in large numbers and in one patient accounted for all isolates of *P. aeruginosa*. Heterogeneity could not be explained on the basis of mixed infections with more than one strain of *P. aeruginosa*. In most patients, mucoid variants of different classes belonged to the same pyocin type.

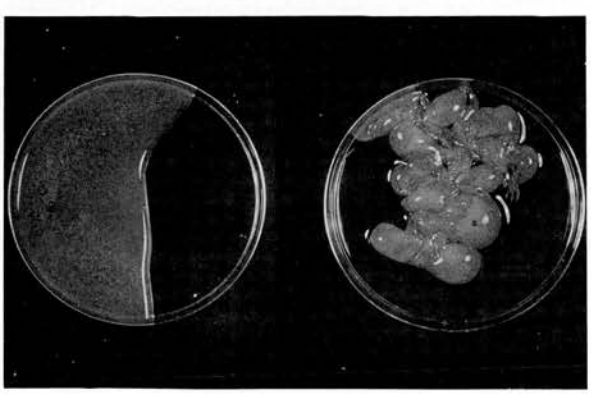

Figure 1. Viscid 1% aqueous solution of alginate extracted from *Pseudomonas aeruginosa* strain PAO 579 (*left*) and the gel that forms (*right*) in the presence of 3-m*M* Ca^{++}.

Pulmonary clearance of P. aeruginosa. We attempted to identify a possible advantage for mucoid *P. aeruginosa* in the respiratory tract by investigating the clearance of isogenic mucoid and nonmucoid bacteria from rat lungs. Table 3 compares the survival of the group 1 mucoid mutant strain PAO 579 or the nonmucoid parent strain PAO 381 of *P. aeruginosa* in homogenized lungs after transtracheal inoculation. No viable *P. aeruginosa* were recovered 24 hr after inoculation with 10^6 cfu of washed or unwashed bacteria, and no animals died. Further experiments showed that few bacteria were recovered after 6 hr. A typical result for lungs sampled at 3 hr and 6 hr indicated that the mucoid strain PAO 579 was cleared less efficiently than the nonmucoid strain PAO 381 ($P < .01$, Student's t test). No significant difference in the clearance rate of strains PAO 579 and PAO 381 was observed when washed cell suspensions were used. Washing significantly increased the clearance of strain PAO 579 ($P < .001$) but not of strain PAO 381.

Table 3. Survival of isogenic mucoid and nonmucoid *Pseudomonas aeruginosa* in rat lungs after transtracheal instillation.

Time after instillation (hr)	Percentage of indicated inoculum recovered*			
	PAO 579 (mucoid)		PAO 381	
	Washed	Unwashed	Washed	Unwashed
3	0.32 ± 0.04	19.3 ± 2.15	0.30 ± 0.03	1.6 ± 0.62
6	. . .	4.1 ± 0.54	. . .	0.27 ± 0.02

* Expressed as percentage of inoculum cfu recovered from lung homogenates. Values are expressed as mean ± SE; each group comprised five animals.

Discussion

It is arguable that in no other infection does *P. aeruginosa* exhibit the complexity and range of biological properties that are observed in chronic pulmonary infection in patients with cystic fibrosis; however, an appreciation of this complexity of such infection is essential not only to an understanding of the natural history of the infection but also to an explanation of the apparent contradictions in the literature that result from generalizations on this subject, in particular those dealing with the nature of the mucoid form.

Mucoid *P. aeruginosa* frequently are assumed to be a homogeneous class of the species possessing basic common properties. Increasingly, however, genetic and physiologic studies have revealed the heterogeneity of this unusual form of the species.

Genetic studies [15, 21] of isogenic mucoid mutants of PAO strains have shown that at least two chromosomal loci are involved in the control of alginate synthesis. Individual mucoid strains isolated in vitro or from patients with cystic fibrosis differ considerably in the amount of alginate produced under defined conditions; in the degree of acetylation of the alginate, which is proportional to viscosity of the polymer; and in the optimal conditions for alginate synthesis [22].

Extreme sensitivity to carbenicillin in *P. aeruginosa* has previously been observed and its association with respiratory isolates noted [23]. The phenomenon frequently is encountered in mucoid strains but is not dependent on alginate production [14].

The significance of heterogeneity in *P. aeruginosa* isolated from patients with cystic fibrosis is further emphasized by the heterogeneous population of mucoid variants found within individual sputum samples from patients with cystic fibrosis. Heterogeneity cannot be explained by simultaneous infection with more than one strain of *P. aeruginosa*.

The nature of heterogeneity reported in this paper involves forms of mucoid *P. aeruginosa* that possess identical colonial appearance when grown on PIA. Other reports [24, 25] have described heterogeneity among strains isolated from individual sputa of patients with cystic fibrosis with regard to colonial appearance (e.g., rough, mucoid, or dwarf) and have noted differences in antibiotic susceptibility associated with these different colonial types. It was suggested [24] that studies of antibiotic susceptibility should be performed on several colonies of each colonial type of *P. aeruginosa* isolated from an individual sputum sample. We would support this suggestion and further emphasize that up to 100-fold differences in susceptibility to β-lactam antibiotics can be found in mucoid colonies, isolated from the same sputum, that are indistinguishable in colonial appearance [14].

Two recent reports have provided valuable insight into the natural history of *P. aeruginosa* infection in patients with cystic fibrosis, in particular the initial selective adhesion and colonization of the upper respiratory tract by nonmucoid strains of *P. aeruginosa* and the subsequent emergence of the mucoid form. Woods et al. [26] reported that in vitro adherence of *P. aeruginosa* to buccal epithelial cells was significantly higher in cells obtained from patients with cystic fibrosis than in those from control subjects. Mucoid *P. aeruginosa* adhered in significantly lower numbers than did nonmucoid forms, and this pili-mediated adherence varied directly with the loss of

protease-sensitive fibronectin from the cell surface and the increased levels of salivary proteases found in patients with cystic fibrosis. In contrast, Marcus and Baker [27] observed that in ciliated hamster tracheal epithelium mucoid *P. aeruginosa* adhered to the cilia and formed mucoid microcolonies, whereas nonmucoid forms showed little or no adherence.

The results reported in this paper on the clearance of *P. aeruginosa* from the lower airways of rats suggest a further selective advantage for the mucoid form. We deliberately used the nonmucoid parent strain PAO 381 as the control for these experiments because of the considerable physiologic and genetic heterogeneity found in nonmucoid revertants [13, 28]. As was previously reported by Blackwood and Pennington [29], we found no significant difference between mucoid and nonmucoid organisms when animals were infected with washed cell suspensions; we did observe a difference, however, with unwashed suspensions. An explanation for these results may lie in the nature of alginate production in *P. aeruginosa*. In contrast to the classic cell-bound capsule of *Klebsiella*, the alginate produced by *P. aeruginosa* in vitro is in the form of a loosely associated slime or peripheral capsule [30]. After centrifugation most of the alginate remains in the culture supernate. In the course of the short experimental period, there would be little prospect for alginate synthesis in vivo.

The results reported here do not establish that alginate per se is responsible for enhanced pulmonary survival of mucoid strains. The ability of pseudomonas alginate to gel in normal pulmonary levels of Ca^{++} is striking. Further studies are required to assess the influence of the characteristically increased levels of Ca^{++} found in patients with cystic fibrosis and the role of cell-free and cell-associated alginate in the clearance of *P. aeruginosa*.

It is difficult to assess the relevance of in vitro experiments and animal studies to human disease. Nevertheless, since a reduced rate of pulmonary clearance for mucoid *P. aeruginosa* has been observed in healthy rats, the selective advantage for the mucoid form might be even greater in the compromised lungs of patients with cystic fibrosis.

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Chromosomal Loci Associated with Antibiotic Hypersensitivity in Pulmonary Isolates of *Pseudomonas aeruginosa*

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492a and 492c were two strains of *Pseudomonas aeruginosa* isolated from the sputum of a patient with cystic fibrosis. The strains were closely related but expressed different antibiograms. 492c was hypersensitive (10–100 times more sensitive than 492a) to the β-lactam antibiotics carbenicillin, methicillin, flucloxacillin, mecillinam and cefuroxime and the non-β-lactam, nalidixic acid. 492c also showed enhanced sensitivity (4–8 times more sensitive than 492a) to chloramphenicol, trimethoprim and novobiocin. 492a and PAO8 expressed similar levels of antibiotic resistance, except for trimethoprim, to which 492a was five times more sensitive than PAO8. Two genes associated with antibiotic hypersensitivity were mapped in the 30 min region of the chromosome, by means of R68.45-mediated plate matings between a Leu$^-$ mutant of 492c and PAO8, followed by transductional analysis using phage F116L. The first of these genes, *blsA1*, was closely linked to *nalB*, and in a PAO background, was associated with hypersensitivity to the β-lactams and a moderate increase in sensitivity to chloramphenicol, trimethoprim, nalidixic acid and novobiocin. A further increase in sensitivity to the latter three antibiotics was associated with the second gene, *tpsA1*, which mapped between *ser-3* and *hisV*. This gene could also be transferred to PAO from 492a, thus 492c could have arisen from 492a *in vivo* following a single chromosomal mutation at the *blsA* locus. Isolation of a *blsA* mutant of PAO969 provided further evidence for this theory.

INTRODUCTION

One of the characteristics most commonly associated with *Pseudomonas aeruginosa* is an intrinsic resistance to antimicrobial agents, in particular, the β-lactam antibiotics. Indeed, this property is generally held responsible for the emergence of *P. aeruginosa* in the last three decades as a clinically important opportunist pathogen.

The development of carbenicillin, the first semi-synthetic penicillin with significant anti-pseudomonas activity, was a significant advance in chemotherapy. However, the minimal inhibitory concentration (MIC) of carbenicillin for *P. aeruginosa* is generally 25–50 μg ml^{-1} (Knudsen *et al.*, 1967), which is beyond the levels attainable in soft tissues, e.g. the lung (Marks *et al.*, 1971).

May & Ingold (1972) reported that strains of *P. aeruginosa* isolated from sputum were often considerably more sensitive to carbenicillin than isolates from other sources. In a study involving 111 sputum isolates from patients with cystic fibrosis (CF), chronic bronchitis and bronchiectasis, they found that 35% of strains were sensitive to 6 μg carbenicillin ml^{-1} and some had MICs as low as 0·7 μg ml^{-1}.

Abbreviations: CEF, cefuroxime; CF, cystic fibrosis; DSTA, Diagnostic Sensitivity Test Agar; NA, nutrient agar; NB, nutrient broth; TP, trimethoprim.

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Berche *et al.* (1979) reported a study on antibiotic susceptibilities of mucoid and non-mucoid strains of *P. aeruginosa*. They compared 47 mucoid with 71 non-mucoid isolates on the basis of sensitivities to 18 antibiotics and concluded that both groups could be divided into two distinct classes on this basis. Class A contained strains significantly more resistant to antibiotics such as the aminoglycosides and tetracycline, than those strains allocated to Class B.

While studying the association of *P. aeruginosa* with chronic respiratory infection in CF patients, we have also reported the isolation of strains unusually sensitive (hypersensitive) to a range of antibiotics including the β-lactams carbenicillin, azlocillin, methicillin and also to trimethoprim (Irvin *et al.*, 1981). The carbenicillin MICs of these strains were <1 μg ml^{-1} and thus comparable with those reported by May & Ingold (1972). In addition, we reported that hypersensitive and 'normal' isolates could be obtained from the same sputum specimen and that these strains were invariably of the same pyocin type, indicating a close relationship.

Recently, there has been an increasing interest in the genetic basis of virulence in *P. aeruginosa* and various potential virulence factors have been mapped using the genetically characterized strain, PAO. For example, several chromosomal genes involved in exotoxin A production have been mapped following isolation of toxin-deficient PAO mutants, and the use of classical genetic techniques (Gray & Vasil, 1981*a*; Hanne *et al.*, 1983). Similarly, a chromosomal gene controlling phospholipase C production has been identified (Gray & Vasil, 1981*b*). Both of these potential virulence factors are normally expressed in PAO, but others may be expressed only following a mutation, for example, alginate production leading to a mucoid phenotype which is so common in *P. aeruginosa* isolated from the sputa of CF patients. Mucoid mutants of PAO have been isolated *in vitro* and two chromosomal loci involved in the control of alginate synthesis have been mapped (Govan & Fyfe, 1978; Fyfe & Govan, 1980; J. A. M. Fyfe & J. R. W. Govan, unpublished).

A third approach to the genetic analysis of virulence involves the transfer of the relevant gene(s) from a clinical isolate into a PAO background. This approach is applicable to both chromosomally-encoded and plasmid-encoded characteristics, and has been used in the genetic analysis of extracellular protease production (Wretlind *et al.*, 1983) and transposable antibiotic resistance (Sinclair & Holloway, 1982).

In the course of studying the intrinsic antibiotic resistance of *P. aeruginosa* strain PAO, various loci associated with this characteristic have been mapped on the chromosome (Mills & Holloway, 1976; Okii *et al.*, 1983; Matsumoto & Terawaki, 1982). Functional aspects of the *P. aeruginosa* outer membrane have been examined using an antibiotic 'supersusceptible' mutant of strain 799 (Zimmerman, 1980; Angus *et al.*, 1982; Darveau & Hancock, 1983). However, genetic analysis of this strain proved unsuccessful (Angus *et al.*, 1982).

The genetic basis of antibiotic hypersensitivity as expressed in clinical isolates of *P. aeruginosa* has not, to our knowledge, been reported. May & Ingold (1972) postulated that the normal resistance to carbenicillin might be plasmid-encoded, and that this plasmid may sometimes be lost during growth in the respiratory tract. However, no evidence was presented to support this hypothesis. The present paper describes the genetic mapping of antibiotic hypersensitivity in the clinical isolate 492c (Irvin *et al.*, 1981) by means of inter-strain crosses with suitably marked PAO recipients, followed by transductional analysis using phage F116L (Krishnapillai, 1971). A preliminary report of this work has been presented (Fyfe & Govan, 1983).

METHODS

Bacteria and bacteriophages. The bacterial strains used in this study are shown in Table 1. Donor strains carrying the plasmid R68.45 were constructed according to Haas & Holloway (1976). Phage F116L (Krishnapillai, 1971) was used for transduction and F116c for strain construction.

Media and cultural conditions. Nutrient broth (NB), nutrient agar (NA), and minimal agar have been described previously (Fyfe & Govan, 1980). Amino acid supplements were added at a concentration of 1 mM. The antibiotics used were carbenicillin (Pyopen; Beecham), methicillin (Celbenin; Beecham), benzyl penicillin (Crystapen; Glaxo), ampicillin (Penbritin; Beecham), flucloxacillin (Floxapen; Beecham), mecillinam (Leo Pharmaceuticals), cefuroxime (Zinacef; Glaxo), cefoxitin (Mefoxin; MSD), tetracycline (Glaxo), trimethoprim lactate (Wellcome), nalidixic acid (Winthrop), novobiocin (Sigma), rifampicin (Roche), streptomycin (Glaxo), gentamicin (Roussel),

Table 1. *Strains of P. aeruginosa used in this study*

Strain	Genotype/Description*	Reference
PAO1	Prototroph, *chl-2*	Holloway (1969)
PAO2	*ser-3*	Isaac & Holloway (1968)
PAO4	*arg-47 pyrB52*	B. W. Holloway collection
PAO8	*met-28 ilv-202 str-1*	Isaac & Holloway (1968)
PAO222	*met-28 trp-6 lys-12 his-4 ilv-226 pro-82*	Haas & Holloway (1976)
PAO969	*proC130*	Rella & Haas (1982)
PAO6002	*met-9011 amiE200 nalB4*	Rella & Haas (1982)
PAO6006	*proC130 nalB9*	Rella & Haas (1982)
PAO6511	*proC130 blsA2*	This paper
PAO6524	*proC130 nalB4* derivative constructed using F116c	This paper
PAO6526	Pro^+ transductant of PAO6511 constructed using F116c	This paper
GMA037	*hisV5037*	Mee & Lee (1967)
492a	Clinical isolate, mucoid, prototrophic, *tpsA*	Irvin *et al.* (1981)
492a Leu^-	Leucine auxotroph isolated from 492a following EMS mutagenesis	This paper
492c	Clinical isolate, mucoid, prototrophic, *blsA1 tpsA1*	Irvin *et al.* (1981)
492c Leu^-	Leucine auxotroph isolated from 492c following EMS mutagenesis	This paper
PAJ1	Prototrophic, *tpsA1 blsA1* recombinant from cross 492c Leu^- R68.45 × PAO8	This paper
PAJ2	Prototrophic, *blsA1* recombinant from cross 492c Leu^- R68.45 × PAO8	This paper
PAJ3	Prototrophic, *tpsA1* recombinant from cross 492c Leu^- R68.45 × PAO8	This paper
PAJ5	*met-28 tpsA1*, recombinant from cross 492c Leu^- R68.45 × PAO8	This paper
PAJ6	*ilv-202 tpsA1*, transductant of PAJ5 using F116c grown on PAO8 met^+	This paper

* Genotype symbols are the same as those used for *E. coli*; *blsA* designates sensitivity to β-lactam antibiotics, *tpsA* sensitivity to trimethoprim, *ami* acetamide utilization and *str* resistance to streptomycin.

tobramycin (Lilley), and kanamycin (Bristol Laboratories). All antibiotics were incorporated in Diagnostic Sensitivity Test Agar (DSTA; Oxoid) at the appropriate concentration.

Broth cultures were grown in McCartney bottles on an orbital incubator (Gallenkamp) at 140 r.p.m., and all cultures were incubated at 37 °C, unless stated otherwise.

Isolation of mutants. Auxotrophic mutants were isolated after EMS mutagenesis (Watson & Holloway, 1976) and carbenicillin enrichment (Watson & Holloway, 1978).

Carbenicillin-hypersensitive mutants were isolated following EMS mutagenesis and screening of colonies by replica plating onto DSTA plates containing 5 μg carbenicillin ml^{-1}.

Antibiotic sensitivity testing. Exponential phase NB cultures were diluted in physiological saline to a concentration of 10^5 organisms ml^{-1}. A multiple inoculator (Mast) was used to dispense samples (containing 10^2–10^3 cells) onto DSTA plates appropriately supplemented with the antibiotics. The MIC was read as the lowest concentration of antibiotic that caused complete growth inhibition after 18 h incubation at 37 °C.

Plate matings. The procedure used was that described by Stanisich & Holloway (1972). Prior to inter-strain matings, NB cultures of the recipient strains were incubated for 18 h at 43 °C (Rolfe & Holloway, 1966).

Transductions. These were performed according to the method of Krishnapillai (1971).

RESULTS

Antibiotic sensitivities

It has previously been reported that 492a and 492c differ in their sensitivities to carbenicillin, azlocillin, piperacillin, ticarcillin, methicillin, tetracycline and trimethoprim (Irvin *et al.*, 1981).

We determined the MICs of an extended range of antibiotics for 492a and 492c, and the results are shown in Table 2. Strain PAO8 was also included to confirm that the MICs obtained for this strain were comparable with 492a and not the hypersensitive 492c. However, 492a was five times more sensitive to trimethoprim than PAO8. Table 2 shows that 492c is generally hypersensitive to the β-lactam antibiotics with the following exceptions: benzyl penicillin,

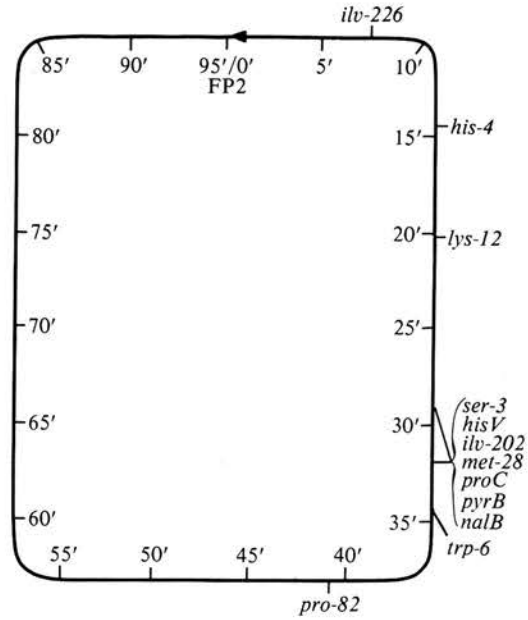

Fig. 1. Genetic map of *P. aeruginosa* showing markers relevant to this study. Based on Holloway & Crockett (1982).

Table 2. *Sensitivities of P. aeruginosa strains 492a, 492c and PAO8 to antibiotics*

Antibiotic Strain ...	MIC (μg ml^{-1}) 492a	492c	PAO8
Carbenicillin	80	0·6	40
Methicillin	>500	10	500
Benzyl penicillin	500	500	>1000
Ampicillin	100	100	200
Flucloxacillin	>500	50	>500
Mecillinam	500	10	500
Cefuroxime	>500	10	400
Cefoxitin	400	400	500
Cephaloridine	500	500	>1000
Chloramphenicol	40	10	60
Tetracycline	10	6	10
Trimethoprim	40	5	200
Nalidixic acid	60	2	80
Novobiocin	>500	100	500
Rifampicin	40	40	20
Streptomycin	40	40	>500*
Gentamicin	0·6	0·6	0·6
Tobramycin	0·4	0·4	0·2
Kanamycin	60	60	60

* PAO8 is resistant to streptomycin due to the *str-1* locus.

ampicillin, cefoxitin and cephaloridine. Increased sensitivity to trimethoprim was confirmed for 492c, and also noted for nalidixic acid and novobiocin. No differences between 492a and 492c were observed for rifampicin, streptomycin, gentamicin, tobramycin or kanamycin.

Transfer of antibiotic hypersensitivity from 492c to PAO

Figure 1 is a chromosome map of *P. aeruginosa* PAO showing the locations of markers relevant to this study. A donor derivative of 492c was prepared by isolating a Leu$^-$ mutant, and

Table 3. *Results of recombinant analysis following a plate mating between 492c Leu⁻ (R68.45) and PAO8*

Selected marker	Percentage co-inheritance of unselected character			
	CEF^{s}*	TP^{s}†	Met^{+}	Ilv^{+}
met-28^{+}	80	82	–	77
ilv-202	75	88	87	–

* CEF^{s} designates sensitivity to 100 μg cefuroxime ml^{-1}.
† TP^{s} designates sensitivity to 100 μg trimethoprim ml^{-1}.

transferring R68.45 into this strain. Plate matings were performed between 492c Leu⁻ (R68.45) and the multiple-marker recipient PAO222. Selection was made for each of the auxotrophic markers and 100 recombinants for each marker were scored for co-inheritance of cefuroxime sensitivity (on DSTA plus 100 μg cefuroxime ml^{-1}). Increased sensitivity to cefuroxime rather than carbenicillin was chosen initially to avoid any problems associated with the inheritance of R68.45 by some of the recombinants (R68.45 codes for a type IIIa β-lactamase which is active against carbenicillin but not cefuroxime). Recombinant analysis revealed that 56% of the *met-28*$^{+}$ colonies were sensitive to 100 μg cefuroxime ml^{-1} and likewise 20% of the *trp-6*$^{+}$ colonies. Less than 1% of the recombinants for the other markers, i.e. *ilv-226*, *his-4*, *lys-12* and *pro-82* had co-inherited this characteristic.

In order to map the locus associated with increased cefuroxime sensitivity more precisely, a plate mating was performed between 492c Leu⁻ (R68.45) and PAO8 (*met-28 ilv-202*). Selection was made for both auxotrophic markers and recombinants scored for both cefuroxime and trimethoprim sensitivity (on DSTA plus 100 μg trimethoprim ml^{-1}). The results of this recombinant analysis are shown in Table 3, and indicate that cefuroxime sensitivity (CEF^{s}) and trimethoprim sensitivity (TP^{s}) do not always cosegregate. All CEF^{s} recombinants were TP^{s}, but a percentage of the TP^{s} colonies were CEF^{r}.

The MICs for representatives of each recombinant class, i.e. CEF^{s} TP^{s}, CEF^{r} TP^{s} and CEF^{r} TP^{r}, were determined using those antibiotics to which 492c is hypersensitive, i.e. carbenicillin, methicillin, flucloxacillin, mecillinam, cefuroxime, chloramphenicol, trimethoprim, nalidixic acid and novobiocin.

All CEF^{r} TP^{r} recombinants (three tested) were indistinguishable from PAO8 on the basis of MICs to these antibiotics. The CEF^{s} TP^{s} group (eight tested) could be subdivided on the basis of their trimethoprim, nalidixic acid and novobiocin MICs. Three recombinants were very sensitive to these antibiotics with trimethoprim and novobiocin MICs of 10 μg ml^{-1}, and a nalidixic acid MIC of 5 μg ml^{-1}. The other CEF^{s} TP^{s} recombinants were moderately sensitive with the following MICs: trimethoprim 60 μg ml^{-1}, novobiocin 50 μg ml^{-1}, nalidixic acid 20 μg ml^{-1}. All eight recombinants were equally sensitive to the β-lactam antibiotics and chloramphenicol, and with the exception of cefuroxime were as sensitive as 492c. The third class of recombinant, CEF^{r} TP^{s} (five tested), displayed moderate sensitivity to trimethoprim, nalidixic acid and novobiocin, with MICs of 60 μg ml^{-1}, 20 μg ml^{-1} and 100 μg ml^{-1}, respectively.

From these results, we postulated that the antibiotic hypersensitive phenotype of 492c is determined by two separate genes, both linked to *met-28* and *ilv-202*, when transferred into PAO8. We propose to call these genes *blsA1*, coding for hypersensitivity to certain β-lactams and a moderate sensitivity to trimethoprim, nalidixic acid and novobiocin; and *tpsA1* coding for increased sensitivity to the latter three antibiotics but not the β-lactams. Table 4 shows the relevant MIC values for PAO8 and three typical recombinant strains PAJ1 (*blsA1 tpsA1*), PAJ2 (*blsA1*) and PAJ3 (*tpsA1*).

Transfer of tpsA1 from 492a to PAO8

As shown in Table 2, the trimethoprim MIC for 492a is fivefold less than for PAO8. In addition, the increased sensitivity of 492c to trimethoprim, compared with 492a (eightfold), is of the same order as the MIC difference between PAJ1 and PAJ3 (sixfold) (see Table 4).

Fig. 2. Approximate locations of *blsA* and *tpsA* on the basis of results obtained from plate matings between 492c Leu$^-$ (R68.45) and PAO8.

Table 4. *Antibiotic sensitivities of PAO8 and typical recombinant strains PAJ1, PAJ2 and PAJ3*

	MIC (μg ml^{-1})			
Antibiotic Strain ...	PAO8	PAJ1 (*blsA1 tpsA1*)	PAJ2 (*blsA1*)	PAJ3 (*tpsA*)
Carbenicillin	40	0·4	0·4	40
Methicillin	500	10	10	500
Flucloxacillin	>500	50	50	>500
Mecillinam	500	10	10	500
Cefuroxime	400	100	100	400
Chloramphenicol	40	20	20	40
Trimethoprim	200	10	60	60
Nalidixic acid	80	5	20	20
Novobiocin	500	10	50	10

On this basis, we proposed that *tpsA1* is present in both 492a and 492c, and that the antibiotic hypersensitivity of the latter strain can be explained by a mutation at the *blsA* locus, i.e. there is only a single gene difference between 492a and 492c accounting for antibiotic hypersensitivity.

Using the mapping procedure already described for 492c, a donor derivative of 492a [492a Leu$^-$ (R68.45)] was plate mated with PAO8, and the *met*$^+$ and *ilv*$^+$ recombinants scored on DSTA containing either 100 μg trimethoprim ml^{-1} or 100 μg cefuroxime ml^{-1}. As expected, a proportion of recombinants failed to grow well on trimethoprim, though all grew well on cefuroxime.

Mapping of blsA and tpsA by transduction using phage F116L

From the plate mating results, it was possible to assign approximate chromosomal locations to *blsA1* and *tpsA1* relative to *ilv-202* and *met-28* (see Fig. 2). However, to map these loci more precisely with respect to other markers in the 30 min region, transductional analysis was performed.

In the case of *blsA1*, a preparation of F116L was grown on PAJ2 and used to transduce recipient strains carrying the auxotrophic markers *met-28* (PAO8), *ilv-202* (PAO8), *proC130* (PAO969) and *pyrB52* (PAO4). Selection was made for these markers and cotransduction of *blsA1* scored on DSTA plus 5 μg carbenicillin ml^{-1}.

In the case of *tpsA1*, transducing preparations of F116L could not be propagated on any recombinants containing this marker, as they did not appear to support vegetative growth. However, *tpsA1* strains retained the receptors for this phage as they were good recipients in F116L-mediated transductions, so in order to map this locus, an *ilv-202 tpsA1* derivative, PAJ6, was constructed (via the intermediate strain PAJ5) and used as recipient in transductions mediated by F116L propagated on either PAO1, PAO2 (*ser-3*) or GMA 037 (*hisV5037*). In each case selection was made for *ilv*$^+$ transductants and these were scored for co-inheritance of *tpsA*$^+$ and where appropriate, the unselected auxotrophic markers. Figure 3 shows the locations obtained for *blsA* and *tpsA*.

Isolation of a blsA mutant of PAO969

Working on the hypothesis that 492c could have arisen from 492a by a single step mutation at the *blsA* locus, we postulated that a similar *blsA* mutant could be isolated from a PAO strain.

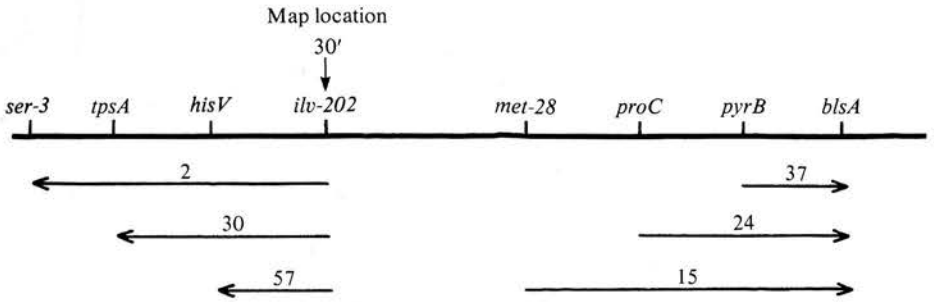

Fig. 3. Mapping of *tpsA1* and *blsA1* by transduction. Values indicate percentage cotransduction using phage F116L (the mean of several experiments scoring at least 200 transductants). Arrowheads point to the unselected marker.

Table 5. *Results of F116L-mediated transductions to determine the relationship between nalB and blsA*

F116L grown on:	Recipient	Percentage cotransduction with *proC*+*		
		blsA2	*nalB4*	Wild-type
PAO1 (*nalB4*+ *blsA2*+)	PAO6511 (*blsA2*)	–	–	33
	PAO6524 (*nalB4*)	–	–	43
PAO6002 (*nalB4*)	PAO6511 (*blsA2*)	–	27	<0·5
PAO6526 (*blsA2*)	PAO6524 (*nalB4*)	19	–	3

* In each case, selection was made for *proC*+ and 200 transductants were scored for the co-inheritance of *nalB4*+/− and *blsA2*+/− on DSTA plus 50 μg and 5 μg carbenicillin ml^{-1}, respectively.

PAO969 was mutagenized with EMS and the potential mutant colonies screened for hypersensitivity to carbenicillin by replica plating to DSTA plates containing 5 μg carbenicillin ml^{-1}. From approximately 5000 colonies screened, one failed to grow on medium containing carbenicillin. Fortuitously, this mutant (PAO6511) exhibited a similar antibiogram to the *blsA1* recombinants, i.e. PAO6511 was highly sensitive to carbenicillin, moderately sensitive to cefuroxime, trimethoprim, nalidixic acid, novobiocin and chloramphenicol, but remained resistant to cefoxitin, cephaloridine and benzyl penicillin. However, PAO6511 was more resistant to methicillin, mecillinam and flucloxacillin than a *blsA1* transductant of PAO969.

F116L transductions revealed 33% cotransduction between *proC* and the mutant locus. Furthermore, when F116L propagated on PAJ2 was used to transduce PAO6511, selecting for *proC*+, <0·5% of the transductants were able to grow on 5 μg carbenicillin ml^{-1}, indicating a very close linkage between *blsA1* and the PAO mutation, which, on this basis, we have designated *blsA2*.

Relationship between blsA and nalB

Rella & Haas (1982) have described a class of mutants (*nalB*) in strain PAO which are resistant to nalidixic acid, novobiocin, carbenicillin and various other β-lactams. We examined two *nalB* mutants PAO6002 (*nalB4*) and PAO6006 (*nalB9*) to determine whether they showed increased resistance to cefuroxime, trimethoprim, methicillin and chloramphenicol. Both strains showed increased resistance to all four antibiotics with the following relative MICs with respect to PAO969: cefuroxime, ×3; trimethoprim, ×6; methicillin, ×5; chloramphenicol, ×10.

nalB has been mapped at 32 min on the PAO chromosome, distal to *pyrB* using G101 transduction (Rella & Haas, 1982), so we determined the location of *blsA2* with respect to this locus, using strains PAO6511 and PAO6002. In order to perform reciprocal transductions, a *proC* derivative of PAO6002 (PAO6524) and a *proC*+ derivative of PAO6511 (PAO6526) were

constructed using F116c. Reciprocal transductions using F116L were performed between the *blsA* and *nalB* strains, in each case selecting for Pro^+ transductants and scoring for *blsA* and *nalB* on DSTA plates containing 5 μg and 50 μg carbenicillin ml^{-1}. The results obtained are shown in Table 5, and suggest that *blsA* and *nalB* are separate, but closely linked loci, the gene order being *proC–nalB–blsA*.

DISCUSSION

Previous genetic studies on the intrinsic resistance of *P. aeruginosa* strain PAO have identified various chromosomal loci associated with this characteristic. Thus we tested the hypothesis that the loss of intrinsic resistance in 492c was due to a chromosomal mutation, rather than loss of a plasmid as suggested by May & Ingold (1972). Inter-strain crosses between 492c Leu^- (R68.45) and PAO8 identified two distinct loci, in the 30 min region of the chromosome, associated with antibiotic sensitivity. The first of these, *blsA*, is associated with sensitivity to the β-lactams carbenicillin, methicillin, flucloxacillin, mecillinam and cefuroxime, and also the non-β-lactams chloramphenicol, trimethoprim, nalidixic acid and novobiocin and cannot be transferred to PAO from 492a.

The isolation of a *blsA* derivative of PAO following EMS mutagenesis provides further evidence that 492c could have arisen from 492a *in vivo* following a single-step mutation which was then selected out in the lung environment. This mutation–selection mechanism is also responsible for the emergence of mucoid strains of *P. aeruginosa* during the course of chronic pulmonary infections in CF patients. All strains of *P. aeruginosa* have the necessary structural genes to synthesize alginate, but these are normally subject to repression. Mutation in a regulator gene close to the major FP2 origin results in derepression of alginate synthesis giving rise to a mucoid strain (Govan & Fyfe, 1978; Fyfe & Govan, 1980; J. A. M. Fyfe & J. R. W. Govan, unpublished).

Three major questions remain to be answered regarding antibiotic hypersensitivity associated with respiratory isolates. Firstly, whether all such isolates have the same mutation as 492c or whether they form a genetically heterogeneous group. We have examined several pairs of strains (i.e. hypersensitive and 'normal' from the same sputum specimen) isolated from different patients on the basis of antibiotic sensitivities and in general, the antibiograms have been similar to 492a and 492c (results not shown). Genetic analysis of one other hypersensitive strain gave identical results to those obtained with 492c, even though this strain was isolated from a different patient and was of a different pyocin type. However, further work is required to determine whether the *blsA* locus is the only one involved in clinically associated antibiotic hypersensitivity.

Secondly, we would like to know the structural basis of this characteristic. We have previously reported that 492c has two extra outer membrane proteins when compared with 492a (Irvin *et al.*, 1981), but further studies have failed to confirm that these are associated with antibiotic sensitivity.

Several studies on the structural basis of antibiotic hypersensitivity in *P. aeruginosa* have been reported (Noguchi *et al.*, 1980; Curtis *et al.*, 1981; Angus *et al.*, 1982; Zimmerman, 1980; Kropinski *et al.*, 1982; Darveau *et al.*, 1983) and these have suggested that increased permeability of the cell envelope is responsible for this characteristic. In the case of 799/61, a 'supersusceptible' mutant of strain 799 (Zimmerman, 1979), alterations in the LPS composition have been detected (Kropinski *et al.*, 1982; Darveau *et al.*, 1983). Similar mutants (*envA*) have been reported in *Escherichia coli* (Grundstrom *et al.*, 1980; Boman *et al.*, 1974).

The close linkage of *blsA* and *nalB* on the *P. aeruginosa* chromosome is interesting in view of the fact that *nalB* mutants are more resistant to those antibiotics to which *blsA* strains are hypersensitive, and it is tempting to speculate that these two loci form part of a gene cluster controlling outer membrane permeability in this organism.

Finally, the clinical and biological significance of antibiotic hypersensitivity remains to be elucidated. Superficially it appears paradoxical that hypersensitive strains arise in patients undergoing aggressive antibiotic therapy, often with β-lactams. The lung, however, is a

notoriously difficult site in which to achieve therapeutic levels of anti-pseudomonas antibiotics, and in this environment the vulnerability of hypersensitive strains may be more apparent than real.

Mutations to increased antibiotic sensitivity are also encountered amongst naturally occurring gonococci (Eisenstein & Sparling, 1978; Lysko & Morse, 1981) and it has been suggested that mucosal surface pathogens may find it advantageous to be flexible with respect to outer membrane structure and that increased permeability may aid colonization in this environment (Lysko & Morse, 1981).

The emergence of antibiotic-hypersensitive strains of *P. aeruginosa* during the course of chronic pulmonary infections, regardless of the selective pressure, provides yet another example of this organism's ability to adapt to a changing environment.

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SYNTHESIS, REGULATION AND BIOLOGICAL FUNCTION OF BACTERIAL ALGINATE

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INTRODUCTION

Historically, interest in bacterial exopolysaccharides has been chiefly medically orientated. The association of capsule production with virulence has been noted in several important bacterial pathogens, the earliest example being Streptococcus pneumoniae (pneumococcus). The virulence of pneumococci for man and animals was found to depend on production of a polysaccharide capsule which surrounds the organism and protects it from phagocytosis. Non-capsulate mutants of pneumococci occur spontaneously and are easily recognised on agar medium as they lack the glossy, mucoid appearance characteristic of wild-type, capsulate strains. These non-capsulate mutants are easily phagocytosed in vivo and are avirulent. Griffiths (ref. 1) showed in 1928 that when a mixture of live, non-capsulate and dead, capsulate pneumococci was injected into mice, some of the mice died of septicaemia and from the blood of these mice, live capsulate pneumococci were isolated. This was the first demonstration of genetic transformation and was thought by Griffith to be mediated by the polysaccharide itself. However, it was later shown by Avery (ref. 2) that DNA was responsible.

Further evidence for the role of capsules as virulence factors was obtained by comparative observations on capsulate and non-capsulate variants derived from the same strain of various other bacterial species.

Examples of bacterial species where evidence has been obtained for the significant virulence-enhancing role of a capsule are Bacillus arthracis, Yersinia pestis, Klebsiella pneumoniae, Haemophilus influenzae and various species of Streptococci. It must be emphasised that capsule production per se is not sufficient to ensure virulence in a bacterial species but it undoubtedly contributes to the overall pathogenic mechanism.

From the earliest studies it became evident that many harmless, saprophytic bacteria, including those found in soil or aquatic environments were heavily capsulate. The role of exopolysaccharides in nature has not been clearly established and is probably diverse and complex. It has been suggested that

they may protect against dessication, phagocytosis and phage attack (refs. 3, 4), or high oxygen tension (ref. 5), participate in uptake of metal ions (refs. 3, 4), as adhesive agents (ref. 6) or ATP sinks (ref. 7) or be involved in interactions between plants and bacteria (ref. 8) or have a possible role in developmental systems such as those found in Myxobacteria (ref. 9).

Industrial microbiologists have become increasingly aware of the commercial potential of procaryotic exopolysaccharides as gelling and emulsifying agents but at the same time are faced with the troublesome consequences of exopolysaccharide production through the formation of bacterial aggregates which reduce fluid flow in a variety of industrial pipe systems. This phenomenon of bacterial adhesion through the agency of exopolysaccharides poses a particular problem in pipe systems which are not readily accessible such as those found in deep water and/or associated with oil recovery. It is fascinating to have observed in the last decade the role of exopolysaccharides in the almost ecumenical development of the various fields of microbiology. Studies by industrial and agricultural microbiologists on the role of exopolysaccharides in bacterial adhesion as an industrial hazard, or as a mechanism of specific adhesion to specific plant hosts, would appear to have re-awakened medical microbiologists to the significant role of exopolysaccharide-mediated adhesion in explaining the localisation of many pathogenic bacteria to specific anatomical sites and surfaces.

The consequences of the virtual explosion of interest in adhesion as a virulence determinant for specific bacterial pathogens have been observed in several well-known bacterial infections. The adherence and localisation of Streptococcus mutans to the surface of teeth following conversion of dietary sucrose to a glucan "cement matrix", and the consequent dental caries which ensues, is only one of many striking examples in medical microbiology of the importance of bacterial exopolysaccharides in localisation and adherence of bacteria to specific human or animal tissues. Knowledge of the determinants of microbial pathogenicity in human, plant and animal hosts is often thwarted by the limitation of in vitro experiments and the necessity to assess results in the context of their relevance in vivo.

The role of exopolysaccharide adhesion in nature and the limitations of in vitro observations were noted by Costerton et al. (ref. 10). The authors drew attention to the fact that certain bacteria cultured for long periods in laboratory conditions lost certain properties normally possessed in their natural environment. Thus microbes that apparently produce no exopolysaccharide in vitro appear to have lost the ability. In nature, the same microbes adhere to a variety of surfaces ranging from the bovine intestine to rocks in fast moving streams by a tangle of fibres of exopolysaccharide that extends from

the bacterial surface to form a felt-like glycocalyx surrounding an individual cell or colony of cells.

On first consideration a precise distinction between the terms capsule and slime as they are presently used and the term glycocalyx might appear semantic and unnecessary. However, it is arguable that the concept of a distinct bacterial glycocalyx and its role in adhesion in vivo has merit. The term glycocalyx may be useful if it defines those cell-associated exopolysaccharides or protein complexes produced in vivo which resemble bacterial capsules or slime but which are lost or difficult to observe in vitro.

Within the wide diversity of microbial exopolysaccharides both in relation to composition and function, bacterial alginate occupies an unusual and fascinating position.

Alginate is a 1, 4 linked copolymer of ß-D-mannuronic acid and its C-5 epimer α-L-guluronic acid (Fig. 1), and is a commercially important polysaccharide with gelling and colloidal properties. It has a variety of uses, e.g. as an ingredient of photographic emulsions and dental impression material and as an additive in various foodstuffs, e.g. ice-cream, jellies and beer (for maintaining a good head).

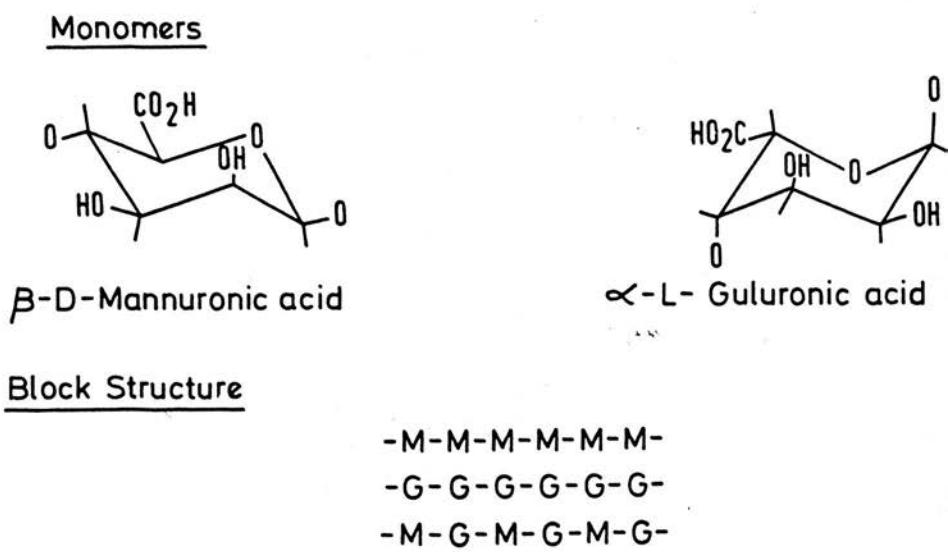

Fig. 1. The structure of alginic acid.

Together with cellulose, hyaluronic acid and sialic acid, alginate is one of the few polymers synthesised by eucaryotic and procaryotic systems. The present source of commercial alginate is eucaryotic namely from marine algae. However, amongst the procaryotes, two bacterial genera are known to contain species capable of alginate production, Azotobacter and Pseudomonas. The present chapter attempts to collate our current scanty knowledge of bacterial alginate in particular its synthesis, regulation and biological significance.

BIOSYNTHESIS AND FUNCTION OF BACTERIAL ALGINATE

The genera Pseudomonas and Azotobacter

Members of the genus Pseudomonas (refs. 11, 12) are common inhabitants of soil, fresh water and marine environments. Some species cause diseases of plants, while others are occasional human and animal pathogens. The cells are gram negative straight or curved rods 0.5-1 μm by 1.5-4 μm and are motile by means of polar flagella. They are strict aerobes, except for those species which can use denitrification as a means of anaerobic respiration. They are among the most metabolically versatile organisms known and are able to utilise a wide variety of organic compounds as sole sources of carbon and energy. The most commonly encountered species belong to the fluorescent group, e.g. P. aeruginosa, P. fluorescens and P. putida. These produce characteristic, water soluble, fluorescent yellow-green pigments. The G + C content of the DNA ranges from 58-70 moles %.

Members of the genus Azotobacter (refs. 11, 12) are also soil and water inhabitants and are able to fix N_2 under aerobic conditions. The cells are gram negative plump rods or cocci, characteristically paired and often containing granules of poly-β-hydroxybutyrate. In young cultures the cells are motile by means of polar or peritrichous flagella. Three species, i.e. A. chroococcum, A. beijerinckii and A. vinelandii produce distinctive resting cells called microcysts which are resistant to dessication. Most strains of this genus characteristically produce large amounts of exopolysaccharide and give smooth glistening colonies on agar medium (Fig. 2). Some strains produce a green fluorescent pigment and the G + C content of the DNA ranges from 63-66 moles %.

The discovery of bacterial alginates

The synthesis of alginate-like polymers by bacteria was first reported in 1964, when Linker and Jones (ref. 13) isolated and partially characterised the exopolysaccharide from a mucoid strain of Pseudomonas aeruginosa (Fig. 3) isolated from the sputum of a patient with cystic fibrosis (CF). They showed using paper chromatography that the substance was a polyuronide with properties

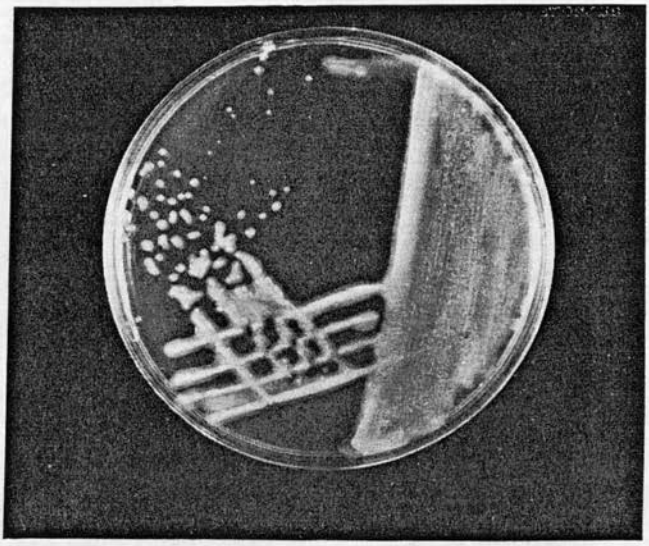

Fig. 2. A strain of A. vinelandii on nitrogen-free medium with glucose.

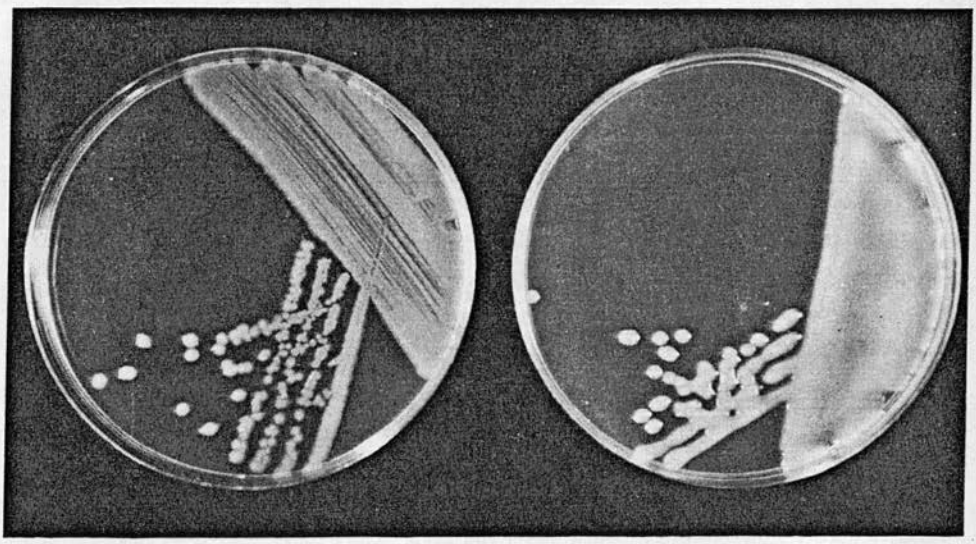

Fig. 3. Clinical isolates of P. aeruginosa grown for 24 h on Difco Pseudomonas Isolation Agar (PIA). On the left, a classical non-mucoid strain and on the right, an alginate-producing mucoid strain.

similar to alginic acid. This finding was confirmed and extended by Carlson and Matthews (ref. 14) in 1966 in a study of 13 mucoid strains of P. aeruginosa from CF and non-CF sources. All were found to produce mannuronic and guluronic acids but the authors were unable to say at this stage whether these were present as homopolymers or as a heteropolymer. Linker and Jones (ref. 15) further analysed the polymer from their strain and demonstrated the presence of acetyl groups as well as confirming the strong resemblance to algal alginate on the basis of composition, structure and physical properties.

In the same year (1966), Gorin and Spencer (ref. 16) demonstrated that the polysaccharide produced by a strain of A. vinelandii was similar in most respects to algal alginate, but was partly acetylated.

Biosynthesis of alginate by A. vinelandii

The production of copious amounts of capsular polysaccharide is characteristic of the majority of strains of A. vinelandii isolated from the environment and the identification of this capsular material as an alginate-like polymer aroused the interest of industrial biochemists interested in developing an alternative source of this useful compound. Such workers sought to elucidate the biosynthetic pathway and the conditions leading to maximal production of alginate (refs. 17-23).

Haug and Larsen (refs. 17, 18) showed that polymannuronic acid is the first polymeric product of alginate biosynthesis produced by vegetative cells of A. vinelandii and that the final polymer contains regions of homo and heteropolymeric blocks, much like the alginate from brown algae.

As part of a project to investigate the commercial feasibility of producing alginate by fermentation, Pindar and Bucke (ref. 19) determined the sequence of reactions involved in alginate biosynthesis from sucrose in A. vinelandii NCIB 9068 by feeding radioactive intermediates to cell-free extracts of the bacteria and by studying the individual enzymes. The pathway previously determined by Lin and Hassid (refs. 24, 25) for the biosynthesis of alginate in the brown algae Fucus gardneri was used as a starting point (Fig. 4).

It should be noted that in this study it was concluded that the epimerisation of mannuronic acid to guluronic acid took place at the monomer level, but in a later study Madgwick et al. (ref. 26) were able to extract a polymannuronic 5-epimerase from brown algae, the presence of which had been hard to prove because the algal enzymes are difficult to extract due to the presence of large amounts of sulphated polysaccharides and phenolic compounds.

The results obtained by Pindar and Bucke for their strain of A. vinelandii suggested the pathway set out in Fig. 5.

No evidence for the formation of free GDP-guluronic acid was found at any stage in the reaction - the intermediate between GDP-mannose and the polymeric

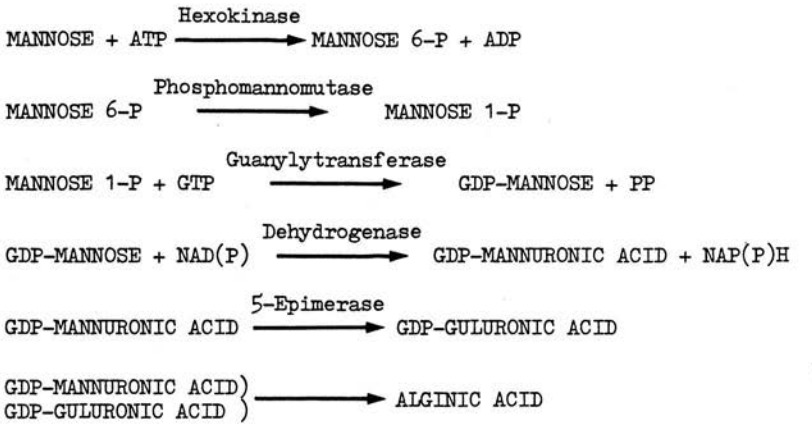

Fig. 4. Biosynthetic pathway for the production of alginic acid from mannose in Fucus gardneri (ref. 25).

material was GDP-mannuronic acid. The composition of the final polymeric product was analysed using partial acid hydrolysis to isolate homopolymeric blocks followed by fractionation at pH 2.85 to separate polymannuronic acid blocks from polyguluronic acid blocks. This procedure indicated that 86% of the polymeric product was in the form of an alternating sequence of mannuronic and guluronic acid units, 10.4% was polymannuronic acid blocks and 3.6% polyguluronic acid blocks, although this composition varied with growth conditions.

The authors did not determine at what stage the acetyl groups were introduced or their association with mannuronic acid or guluronic acid units. The physical location of the polymannuronate epimerase was also to be determined.

Davidson et al. (ref. 27) later showed that the acetyl groups were associated with mannuronic acid residues and suggested that they may play a role in protecting certain of these residues from epimerisation.

An investigation of the physiology of alginate production by A. vinelandii NCIB 9068 (ref. 20) revealed that in batch culture, under phosphate-deficient conditions, alginate synthesis occurred during growth and ceased when the cells entered stationary phase. In a continuous culture system under phosphate limitation the rate of alginate synthesis was independent of specific growth rate. However, by manipulating the conditions of fermentation, e.g. by altering the calcium ion concentration, the molecular weight and viscosity of the product could be varied. Likewise, when growth was limited by a variety of nutrients (ref. 21) including carbon source (sucrose), the rate of alginate synthesis varied only slightly. The only exception was oxygen limitation which was detrimental to alginate synthesis. In addition, when oxygen

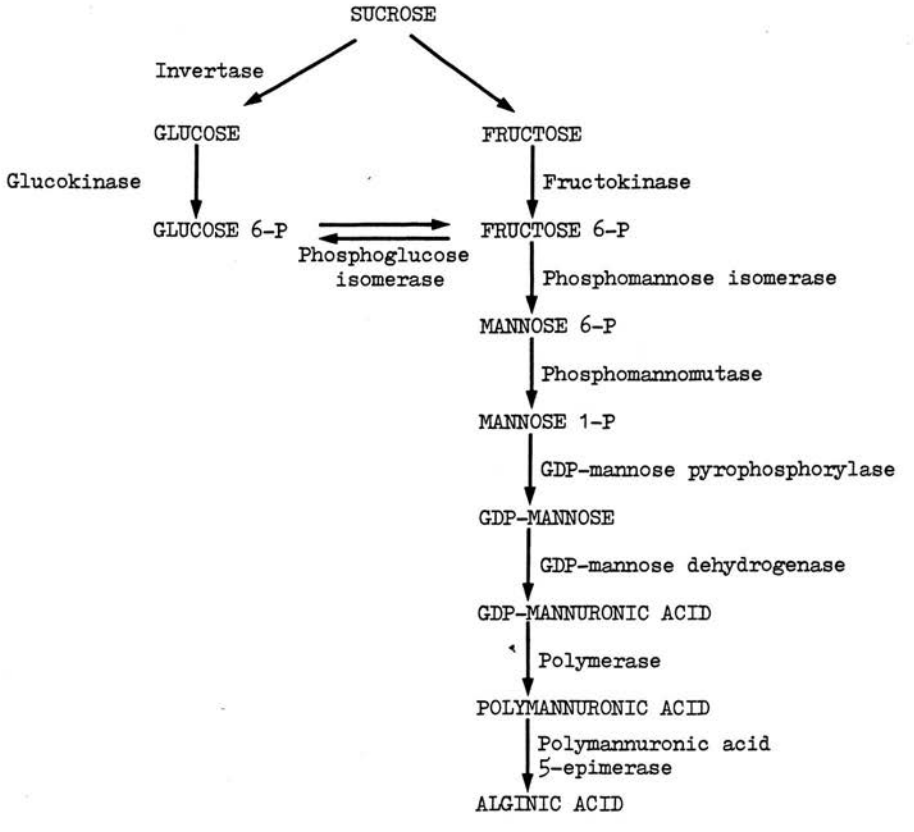

Fig. 5. Pathway of biosynthesis of alginic acid in A. vinelandii (ref. 19).

availability was increased by altering the fermenter agitator speed, the specific respiration rate went up and the conversion efficiency for sucrose into alginate fell from an optimum of 40% to 8% (ref. 22).

In some bacteria, exopolysaccharide synthesis appears to share common precursors and co-factors with cell wall synthesis (ref. 9), hence competition for these intermediates would take place and rate of polysaccharide synthesis would be influenced by growth rate. However, the apparent independence of the rate of alginate synthesis from specific growth rate in A. vinelandii 9068 suggests that this system may not operate in this case (ref. 22).

A later study (ref. 23) using a mutant of strain 9068 with enhanced ability to synthesise alginate, showed that in this strain, alginate production continued after the cessation of growth. Activities of the enzymes phosphomannose isomerase, GDP mannose pyrophosphorylase and GDP mannose dehydrogenase were found to correlate with the amount of alginate produced.

Sucrose was found to be the best carbon source in terms of alginate/ bacterial mass ratio and no alginate was produced when mannose or sorbitol were used as the carbon source. The authors concluded that the biosynthesis of alginate is controlled through the specific activities of the enzymes involved, by an unknown mechanism. No significant regulation through feedback inhibition appears to exist.

Alginate production and cyst formation in A. vinelandii

Concurrent with the studies on the biosynthesis of alginate in A. vinelandii described in the previous section, Sadoff and his colleagues were investigating the cellular differentiation cycle leading to encystment and germination in this organism (refs. 28-33). The life cycle of A. vinelandii, as observed by light microscopy is illustrated in Fig. 6.

Cysts are metabolically dormant cells which are more capable of withstanding dessication than vegetative cells and are viable in the laboratory

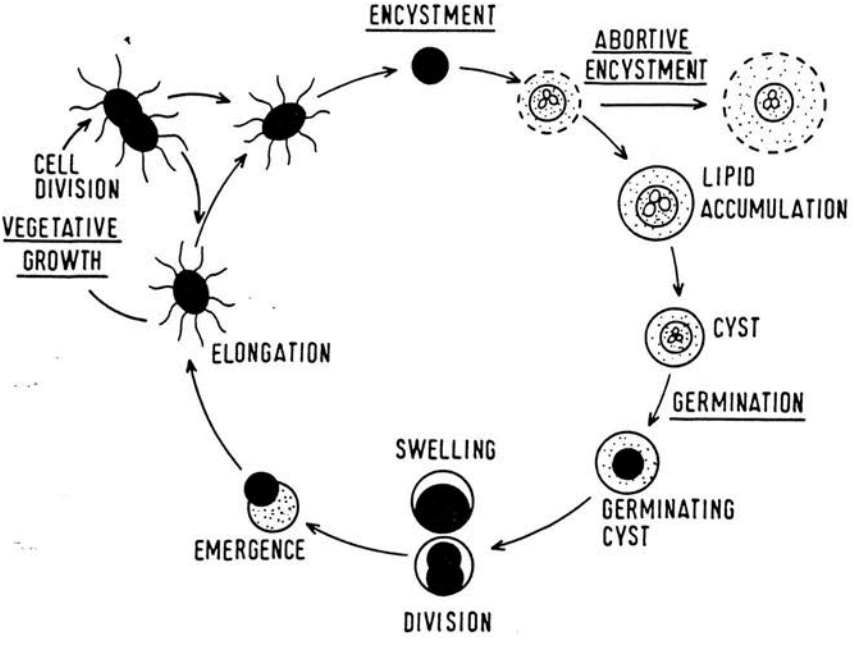

Fig. 6. Schematic diagram of the life cycle of A. vinelandii taken from Sadoff (ref. 33).

for at least 10 years kept in dry soil. They are also more resistant to radiation and sonication than vegetative cells. However, unlike the endospores of gram positive organisms, cysts are not heat resistant.

In 1968, Lin and Sadoff (ref. 28) reported the association of a viscous polymer with the encystment process of A. vinelandii ATCC 12837. Two other groups (refs. 34, 35) later published electronmicrographs of cysts stained with ruthenium red revealing polysaccharide capsular material external to the exine (outer coat) region (Fig. 7). Densely stained material along with unstained regions were seen in the vicinity of the exine, while the intine (inner coat) was also heavily stained.

The relationship of capsule production and cyst formation in A. vinelandii ATCC 12837 was demonstrated by Eklund et al. (ref. 36) using three independent methods.

1. When a phage-induced polysaccharide depolymerase (capable of depolymerising the capsular material) was added to encystment medium, the cells were unable to form typical cysts, exine coats were partially destroyed and intine was greatly reduced.

2. Under conditions unfavourable to polysaccharide production, i.e. when ammonium nitrate was present in the culture medium, encystment did not take place.

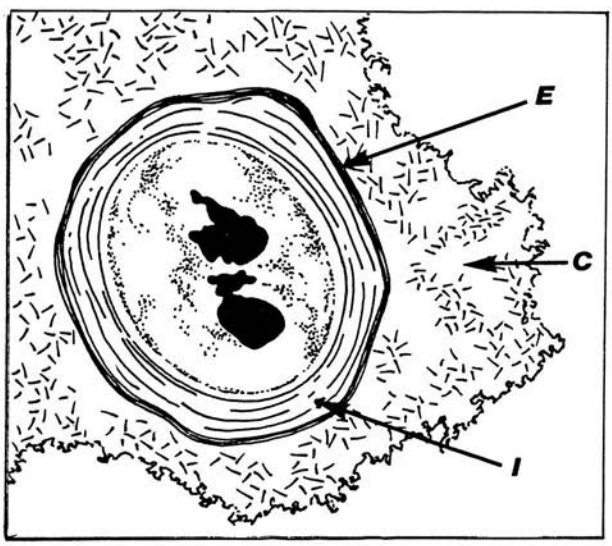

Fig. 7. Diagram of a ruthenium red stained cyst of A. vinelandii based on an electronmicrograph (ref. 34). C, capsular material, E, exine coat, I, intine coat.

3. Non-capsulate mutants of A. vinelandii 12837 were unable to form cysts.

Hence, although capsule production per se is not necessarily followed by encystment, it is a mandatory requirement for encystment to occur.

Chemical analysis of cyst fractions revealed that the intine and exine are rich in protein, carbohydrate and lipid and contain high levels of calcium and lesser amounts of magnesium (ref. 30). It had also been noted that the suspension of mature cysts in EDTA led to the disruption of the exine layer (refs. 29, 37).

Page and Sadoff (ref. 32) examined the role of cations in the structure and integrity of mature cysts and the role of calcium in the encystment process. As part of this study, cyst fractions were examined for the presence of uronic acids in the form of alginate and for polymannuronic acid 5-epimerase activity. Mannuronic and guluronic acids in sequences characteristic of alginate were demonstrated in both the intine and exine cyst fractions. Proportions of the two uronic acids were found to differ between the two fractions - the exine was notably rich in polyguluronic acid whereas the intine was richer in polymannuronic acid, both contained approximately 50% hetero-polymeric blocks. The composition of the intine material resembled the vegetative cell capsular material in block composition and mannuronic/guluronic acid ratio. When calcium ions were omitted from the growth medium, abortive encystment took place, i.e. the central bodies of the cysts were normal but a viscous slime, rich in polymannuronic acid was produced rather than the intine and exine coats. Polymannuronic acid 5-epimerase activity was detected in the mature cyst central body and the culture fluid. This activity was stimulated by the addition of calcium to the calcium-free encystment culture fluid, suggesting that calcium was not required for the production of the enzyme per se, rather for its activity.

The authors postulated that during encystment, polymannuronic acid is initially secreted by the cell. In the presence of calcium, extracellular polymannuronic 5-epimerase converts a large proportion of these residues to guluronic acid. Guluronic acid residues have a high calcium binding capacity (ref. 38) and alginate containing a significant amount of guluronic acid forms a gel in the presence of calcium ions due to the formation of inter-chain linkages. Alginate in this gel form has an even higher selectivity for calcium ions. Hence the coalescence of the exine coat would reduce the availability of calcium for epimerisation of the intine coat, so the proportion of guluronic acid in this fraction would be lower.

The role of the exine coat would appear to be the maintenance of cyst rigidity and resistance to dessication. In addition, the properties of radiation and sonication resistance are dependent on an intact exine (ref. 33). The function of the intine layer is less obvious, however, during germination

there is a gradual loss of its ability to react with electron dense fixatives and stains (ref. 39) suggesting that the contents of the intine may be used as growth substrates.

Alginate-producing strains of P. aeruginosa

The increasing interest during the last decade in alginate-producing strains of P. aeruginosa has arisen for reasons which are in striking contrast to alginate synthesis in A. vinelandii. As already indicated, alginate production is characteristic of the majority of strains of A. vinelandii. In contrast, alginate production in P. aeruginosa is extremely uncommon in strains isolated from a wide variety of human, animal and environmental sources (ref. 40). The important exception to this rarity is the frequent emergence of alginate-producing P. aeruginosa in the lungs of CF patients. The significance of this now classic association in CF is that the increasing predominance of alginate-producing pseudomonas in the lungs is paralleled with clinical deterioration, increasing refractoriness to treatment with antibiotics and a poor prognosis. Unfortunately, concurrent with the increased importance attributed to alginate-producing P. aeruginosa in the last decade, there has arisen considerable confusion in the literature regarding the nature of pseudomonas exopolysaccharide, in particular the distinction between alginate and the more general term slime which historically has been used to describe the viscid liquid phase associated with broth cultures of P. aeruginosa grown under particular conditions. For the purpose of clarity, an attempt will be made to rationalise the use of the terms mucoid, slime and alginate in the light of present knowledge.

The initial recognition of a bacterial characteristic is often made at the colonial level. In 1969, Phillips (ref. 41), described six colonial types of P. aeruginosa, viz., typical, coliform-like, rough, rugose, mucoid and dwarf. The mucoid form (Fig. 3) which produced a large gelatinous colony within 24 h at 37°C on blood agar and which was shown to be associated with alginate production (ref. 13) was observed in only two of the 128 isolates of P. aeruginosa. The single representative of the dwarf type arose as a variant from one of the mucoid isolates.

Mucoid strains of P. aeruginosa have been known to microbiologists since their first reported isolation in 1927 by Sonnenschein (ref. 42). Until the 1960's however, only a few further isolations were reported (refs. 43, 44). The relative rarity of mucoid P. aeruginosa prior to the 1960's could be explained by their colonial resemblance to Klebsiella aerogenes and their misdiagnosis as this species. In the case of blood agar and MacConkey agar cultures this misdiagnosis is undoubtedly still being made, partly because of the poor production of the characteristic pigments pyocyanin and fluorescein

in young cultures of mucoid P. aeruginosa, and further compounded by the poor pigmentation associated with growth on these common diagnostic media. An alternative explanation is that the rarity of mucoid P. aeruginosa was genuine. Today, mucoid strains are almost solely isolated from the sputum produced in chronic respiratory infections in CF patients and indeed the presence of mucoid P. aeruginosa has been suggested as a diagnostic feature of the disease in older, previously undiagnosed patients (ref. 45). In 1948, when Henriksen (ref. 44) described the isolation of a single mucoid strain, the life expectancy of a CF patient was measured in months, today many patients survive into adulthood. The increased isolation of mucoid P. aeruginosa reported by Elston and Hoffman in 1967 (ref. 46) most probably resulted from the significant improvement in management and antibiotic therapy leading to a longer survival and a greater reservoir for potential isolation of these strains.

Elston and Hoffman (ref. 46) noted that capsulation of mucoid P. aeruginosa was not a constant finding, that when seen by the India ink technique (ref. 47) capsules were irregular, indistinct and shown by only some of the individual cells in a culture. We have observed that the presence or absence of a discrete capsule for some strains is dependent on the level of Ca^{++} ions in the growth medium. As is the case with A. vinelandii (ref. 48), growth of the alginate-producing P. aeruginosa 492a (ref. 49) in low levels of calcium resulted in release of the exopolysaccharide from the cells. On centrifugation, this remained in the supernatant fraction and the cells appeared non-capsulate. However, growth in medium containing 1 mM Ca^{++} resulted in the formation of discrete capsules which remained attached to the cells even after washing. This finding may have some relevance to the clinical situation as the bronchial secretions of CF patients contain elevated levels of calcium.

The pioneering work of Doggett and colleagues (refs. 40, 50, 51) in the 1960's which first revealed the association of mucoid P. aeruginosa and CF was accompanied by studies on the composition and properties of the exopolysaccharide which by the end of the decade had been identified as an acetylated heteropolymer of mannuronic and guluronic acids (refs. 13-15, 52) resembling algal alginate and similar to the polymer found in A. vinelandii (ref. 16).

From the earliest studies on P. aeruginosa, classic non-mucoid strains belonging to Phillips colonial types 1 to 4 (ref. 41) have been known to produce viscid, slimy broth cultures particularly when incubation is prolonged and in medium with a high carbon content. Indeed, slime production is so characteristic of the species that its synthesis in medium containing 4% potassium gluconate has been long used as a diagnostic feature (refs. 41, 53). Unfortunately, in recent years, the terms mucoid material and slime have been used indiscriminately and synonymously and considerable ambiguity can be found

in the literature (ref. 54). This confusion is further compounded by a lack of uniformity in the results of analyses carried out on slime extracted from non-mucoid P. aeruginosa (Table 1).

TABLE 1

Analysis of extracellular slime from non-mucoid strains of P. aeruginosa

Strain	Components	Reference
OSU 64	mannose, DNA, RNA, protein	55
B1	mannose, glucose, rhamnose, galactose, glucosamine, galactosamine, glucuronic acid, DNA, RNA	56, 57
OSU 64 NCTC 6750, 7244, 8203, 1999	mannose, glucose, rhamnose, glucosamine, glucuronic acid, hyaluronic acid, DNA, RNA, protein	58
36 clinical isolates	mannose, glucose, fucose, galactose, ribose, rhamnose, glucosamine, galactosamine	59

A review of the literature indicates that whilst pseudomonas slime undoubtedly exists and plays a part as a virulence determinant (refs. 57, 60), the exact composition of this loosely-defined material is extremely variable and dependent upon the strain, the cultural conditions and the method of analysis. For the purpose of this chapter the term slime is restricted to the viscid material consisting of DNA, protein and various polysaccharides which is produced characteristically by all strains of P. aeruginosa. The term mucoid is restricted to those strains producing the characteristic colonial type 5 of Phillips within 24 h on agar media and producing an acetylated alginate-like heteropolymer of mannuronic and guluronic acids.

The isolation of mucoid P. aeruginosa in vivo and in vitro

The association of mucoid strains of P. aeruginosa with chronic pulmonary infection in CF patients is well recognised (refs. 40, 50, 61-63). Evidence for their emergence in vivo was first provided by Doggett et al. (ref. 50) who observed that in individual CF patients, initial colonisation is by non-mucoid P. aeruginosa but, during the course of infection, mucoid isolates gradually emerge and eventually come to predominate. In addition, when the two forms are found in the same specimen, they belong to the same serotype (ref. 63) and pyocin type (ref. 64).

In contrast to their emergence in vivo, when cultured in vitro, mucoid strains of P. aeruginosa tend to revert to the non-mucoid form. Zierdt

and Schmidt (ref. 65) reported that serial transfer on solid medium gave rise to the rapid loss of the mucoid character.

The first report of *in vitro* isolation of mucoid variants was that of Martin (ref. 66) who, during the course of phage typing, noted rings of slimy, mucoid growth around areas of phage lysis, while the background lawn remained non-mucoid. Subculture from these slimy areas resulted in pure cultures of mucoid colonies which looked and behaved like clinical isolates.

These mucoid variants had the same phage type and serotype as the non-mucoid parent and thus were sensitive to the phage responsible for their emergence. From this, Martin concluded that the mucoid variants had not been selected from the non-mucoid population, but rather the presence of phage in the lytic cycle was necessary for the continued expression of the mucoid character. The term "pseudolysogeny" was used to describe this phenomenon. Martin postulated that phage could also be responsible for the emergence of mucoid strains *in vivo*, the source of which would be other strains of *P. aeruginosa*, but no evidence for such mixed infections was presented.

An investigation of the influence of various substances on the stability of mucoid *P. aeruginosa* *in vitro* (ref. 67) revealed that clinical and phage-derived strains maintain their mucoid colonial form when serially subcultured on desoxycholate citrate agar, or in sodium deoxycholate broth. However, the genetic basis for the emergence of the mucoid characteristic remained obscure.

In 1978 we published an alternative method for isolating mucoid variants *in vitro* without the use of phage (ref. 68). This method was based on the observation that mucoid strains are slightly more resistant to some antibiotics than the related non-mucoid forms (ref. 69). The selective agent of choice is carbenicillin and the isolation procedure involves the following steps: (Fig. 8).

1. Approximately 10^7 cells from an 18 h nutrient broth culture of *P. aeruginosa* are spread on to the surface of nutrient agar plates containing carbenicillin at a concentration of 1.5 times the MIC of the strain.

2. After 18 h incubation at 37°C, the resistant colonies are replica plated from the antibiotic containing medium to PIA plates containing no antibiotic.

3. After 18 h incubation at 37°C, the mucoid variants can be recognised as distinctive, watery colonies against a background of non-mucoid growth (Fig. 9).

The frequency of isolation of mucoid variants from *P. aeruginosa* strain PAO using this procedure is approximately 1 in 10^7 cells and this can be increased 40-fold following mutagenesis with ethyl methanesulphonate (EMS). These mucoid variants do not require the continued presence of the antibiotic for mucoid colonial growth and infra-red spectroscopy of the exopolysaccharide has indicated an acetylated polymer of mannuronic and guluronic acids.

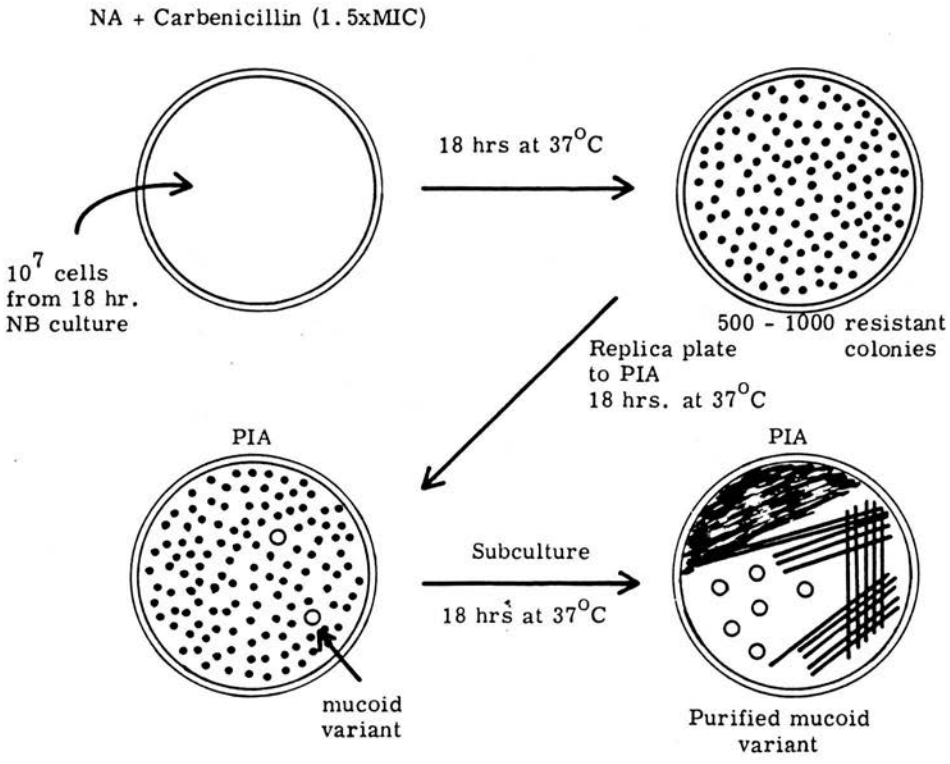

Fig. 8. Technique for the isolation of mucoid variants of P. aeruginosa in vitro by selection for resistance to carbenicillin (ref. 68).

Carbenicillin is not the only antibiotic which can be used to select such mucoid variants - other penicillins and aminoglycosides were also used successfully at a concentration of 1.5 times the MIC for the non-mucoid strain.

The ability to isolate mucoid derivatives of well characterised strains and maintain them in vitro has led to progress in the physiological and genetic studies of alginate synthesis in mucoid P. aeruginosa. In addition, a modified version of the selection procedure has been used to isolate alginate-producing variants of P. putida, P. fluorescens and P. mendocina, in which species alginate biosynthesis had not previously been reported (ref. 70).

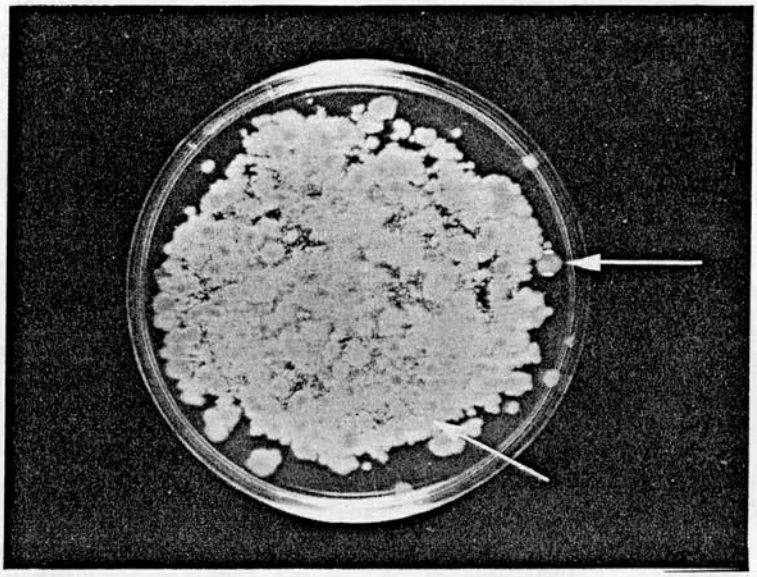

Fig. 9. Isolation of mucoid mutants in vitro following carbenicillin semi-selection. PIA plate showing two mucoid colonies (indicated by arrows) amongst a background of non-mucoid growth.

Biosynthesis of alginate by mucoid P. aeruginosa

Early studies (refs. 13, 15) which identified alginate from mucoid P. aeruginosa employed clinical isolates cultured on Oxoid sensitivity test agar for 24 h at 37°C. The first investigation in which the effect of growth conditions on pseudomonas alginate biosynthesis was examined was that of Evans and Linker (ref. 52). These authors grew three mucoid clinical isolates of P. aeruginosa on various agar media and incubated at 12°C, 25°C or 37°C. Determination of maximal exopolysaccharide production was subjective, i.e. by visual estimation of the ratio of transparent material to the amount of opaque cellular material of a colony and the amount of bacterial exopolysaccharide observed by the India ink technique (ref. 47).

For each strain, a lower incubation temperature resulted in more exopolysaccharide per cell, regardless of growth medium. Exopolysaccharide production was enhanced by growth on MacConkey plates containing sodium chloride or glycerol. Large batches of exopolysaccharide for analysis were obtained by seeding the organisms on to MacConkey agar supplemented with 3% glycerol and incubating at 25°C for four days.

Analysis of the material obtained in this way revealed that different strains varied widely according to the mannuronic/guluronic acid ratio of the exopolysaccharide. However, organisms isolated from the same patient at different

times appeared to produce similar polymers. All the polymers were acetylated and the acetyl content was proportional to the mannuronic acid content. Molecular weights of all the pseudomonas alginates were higher than those of the algal alginates tested.

A later investigation of the biosynthesis of alginate in batch culture (ref. 71) by a mucoid P. aeruginosa, isolated from a CF patient, showed that in a glucose-yeast extract medium, exopolysaccharide was produced throughout the growth phase and ceased simultaneously with growth as a result of glucose exhaustion. In continuous culture, under nitrogen limited conditions, both cell and exopolysaccharide concentrations were largely independent of dilution rate. Polysaccharide was produced under all nutrient limitations tested including carbon limitation.

An early observation made in our own laboratory in studies of mucoid, alginate-producing mutants isolated in vitro from strain PA0381 (ref. 72) was that certain mutants appeared to produce exopolysaccharide on both minimal and complex media, e.g. PA0579, whilst other mutants, e.g. PA0568 failed to produce exopolysaccharide on minimal medium (ref. 73). On this basis, mucoid strains can be classified into groups 1 and 2 where PA0579 represents group 1 and PA0568, group 2. Fig. 10 shows the colonial appearance of PA0568 and PA0579 after 24 h incubation at 37°C on PIA and minimal agar.

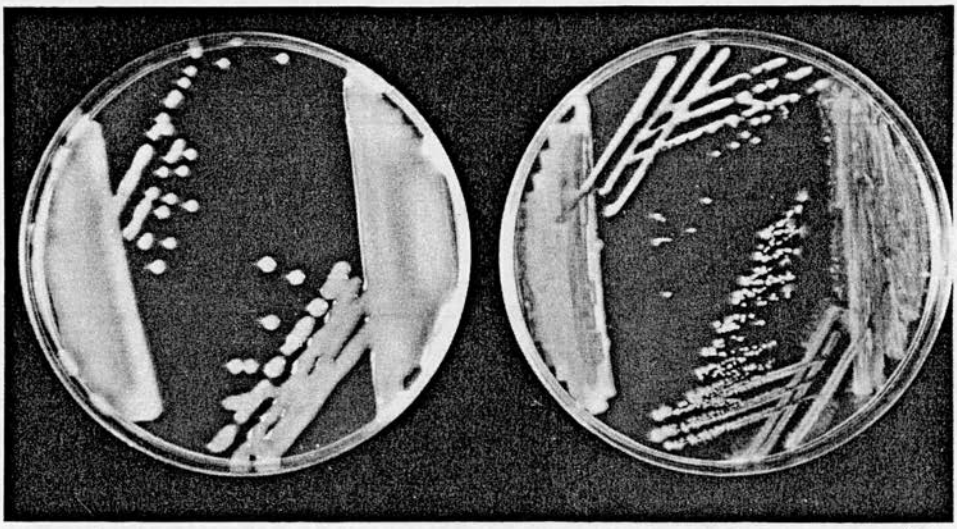

Fig. 10. Two mucoid mutants PA0568 and PA0579 after 24 h incubation at 37°C on PIA (left plate) and minimal agar (right plate). The group 1 mutant PA0579 on the left of each plate produces alginate on both media, whereas the group 2 mutant PA0568 on the right of each plate appears non-mucoid on the minimal agar.

We have also observed that mucoid P. aeruginosa isolated from the sputa of patients with CF can also be classified into these two groups, and in many patients, both forms are isolated simultaneously from the same specimen (ref. 74).

Piggott (ref. 75) studied alginate biosynthesis in a number of mucoid mutants derived from PA0381, including PA0568 and PA0579. In batch culture, in yeast-extract medium with gluconate, polymer production in the majority of strains was not growth associated. In addition, the conditions for optimal alginate production varied markedly between strains. Some strains produced alginate equally well at 30°C and 37°C, whereas others produced best at 37°C. When the composition of the polymers from these strains (collected after 48 h growth in yeast extract medium with 2% gluconate) was analysed, all samples had a high mannuronic acid content ranging from 75% to 95%. The degree of acetylation and viscosity also varied significantly and a linear relationship was observed between % acetylation and viscosity over the range 2.3-8.6% acetate content. In every aspect of alginate biosynthesis studied, the mutants varied considerably although all were derived from the same parent.

Piggott et al. reported enzyme analyses on PA0381 and four of the mucoid mutants (ref. 76). The results obtained for three enzymes, phosphomannose isomerase, GDP-mannose pyrophosphorylase and GDP-mannose dehydrogenase involved in the alginate biosynthetic pathway are shown in Table 2.

TABLE 2

Enzyme levels in P. aeruginosa PA0381 and four mucoid derivatives. Results are expressed in μmoles product formed mg protein^{-1} min^{-1} (ref. 76).

	PA0381	PA0579	PA0578	PA0568	PA0585
Phosphomannose isomerase	22	380	360	280	41
GDP-mannose pyrophosphorylase	6	21	23	9	33
GDP-mannose dehydrogenase	1	17	6	2	6

The low levels of activity of the GDP-mannose metabolising enzymes in PA0568 were increased by the addition of 0.5 μM fluoride indicating the probable presence of a nucleotide hydrolysing enzyme. Attempts to isolate an epimerase from these strains were unsuccessful.

Similar enzyme analyses of four non-mucoid revertants of PA0579 revealed no detectable GDP-mannose pyrophosphorylase or GDP-mannose dehydrogenase while phosphomannose isomerase levels remained elevated.

GENETICS OF BACTERIAL ALGINATE BIOSYNTHESIS

Genetics of P. aeruginosa and A. vinelandii

Genetic analysis of any organism is dependent on (a) the ability to obtain and recognise stable genetic variation both natural and induced, and (b) a system of gene transfer and recombination.

For many years, bacterial genetics was synonymous with Escherichia coli genetics following the pioneering work of Lederberg and Tatum (refs. 77-79). Interest in the genetics of organisms other than E. coli is more recent. The genus Pseudomonas attracted the attention of geneticists because of its biochemical diversity and significance as an opportunist pathogen. Gene transfer by means of conjugation was first described in P. aeruginosa in 1956 (ref. 80), transducing phages were also isolated and characterised (ref. 81) and genetic mapping studies ensued. Most of these studies have used two strains PAO and PAT originally described by Holloway (ref. 82). Strain PAO was isolated from a patient in Australia in 1954. Strain PAT was originally isolated in South Africa in 1950 and carried the sex factor now known as FP2. Several extensive reviews reporting the progress in genetic analysis of P. aeruginosa have been published (refs. 83-87).

For over 20 years, mapping of the PAO chromosome was hampered by the inability to demonstrate genetic circularity. Unlike F, the original E. coli sex factor, which can mobilise the chromosome from many different sites, FP2 apparently had a single origin of transfer, so that accurate mapping of markers by plate and interrupted mating techniques was only possible for the 0' to 40' region (ref. 87).

In strain PAT, however, several R plasmids were found that exhibited chromosome mobilising ability (Cma) (ref. 88). Mapping data obtained using the plasmids FP2, R91-5 (Inc. P-10) and R68 (Inc. P-1) together with transductional analysis, allowed a genetically circular linkage map to be constructed for this strain (refs. 89, 90). The isolation of plasmid R68.45, a variant of R68 with efficient Cma for strain PAO (refs. 91, 92, for review see ref. 93) proved a major step forward in PAO genetics, as it was shown to mobilise the chromosome from multiple origins. Subsequently, a genetically circular map of the PAO chromosome was constructed (ref. 94, Fig. 11). Indeed, because of its broad host range, R68.45 has been a useful tool in the genetic analysis of various other bacterial species including Rhizobium leguminosarum (ref. 95), R. meliloti (ref. 96), R. trifolii (ref. 97) and Rhodopseudomonas spharoides (ref. 98).

Genetic studies of A. vinelandii have had an even more chequered development. Several workers have reported the isolation of auxotrophs, (refs. 99-101), antibiotic resistant mutants (ref. 100) and mutants unable to fix nitrogen (refs. 101-103), but certain other kinds of mutants have proved extremely

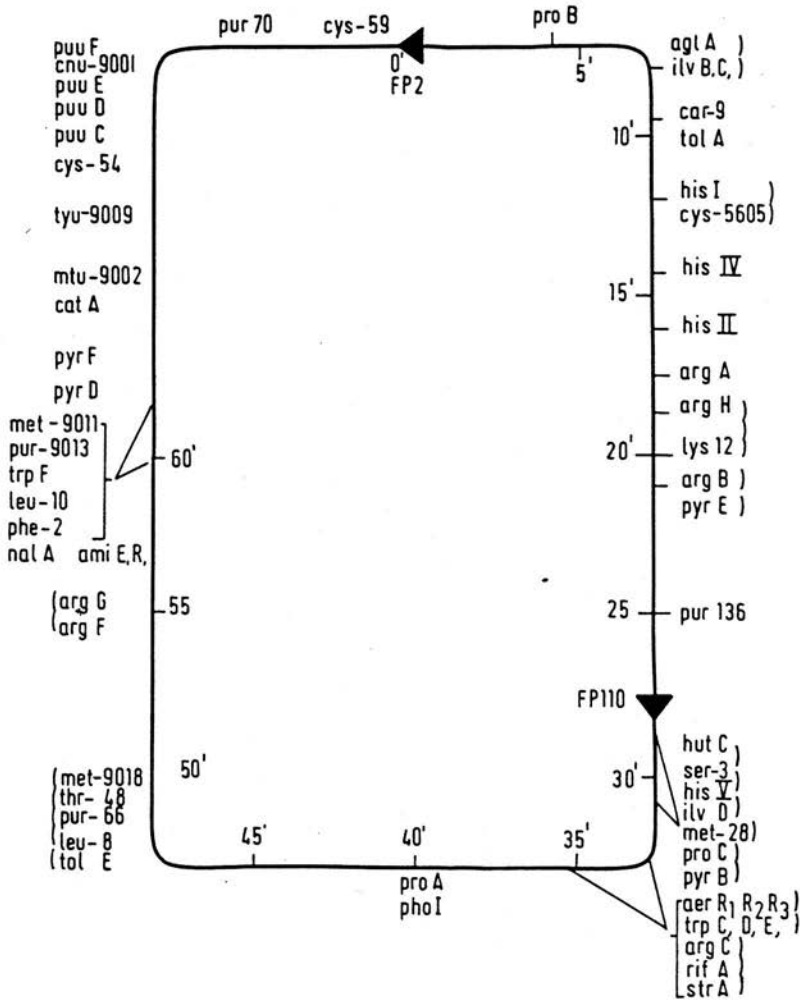

Fig. 11. Chromosome map of P. aeruginosa PAO (ref. 94). Markers whose location is indicated by a bar joined to the map were located by interrupted matings using FP2 donors. Round brackets indicate that markers are cotransducible with one or more of the phages F116, F116L, G101 and E79. Marker abbreviations are as follows:- Anabolic markers: arg, arginine; car, carbamoylphosphate synthase; cys, cysteine; his, histidine; ilv, isoleucine valine; leu, leucine; lys, lysine; met, methionine; phe, phenylalanine; pro, proline; pur, purine; pyr, pyrimidine; ser, serine; thr, threonine; trp, tryptophan. Catabolic markers: ami, amidase; cat, catechol; cnu, carnosine; hut, histidine; mtu, mannitol; puu, purine; tyu, tyrosine. Resistance markers: agl, aminoglycoside; nal, naladixic acid; rif, rifampicin; str, streptomycin. Other markers: aer, aeruginocin production; pho, alkaline phosphatase; tol, aeruginocin tolerant.

difficult to isolate, e.g. those blocked in β-hydroxybutyrate metabolism (ref. 104). The reasons for this are not completely understood, but genetic instability of the mutant DNA is thought to be responsible rather than a problem with mutagenesis per se (ref. 105). It has been estimated that a mid log phase A. vinelandii cell contains enough DNA to account for approximately 40 chromosomes (of the size found in E. coli) (ref. 104). Hence, many of the genes may exist in multiple copies. Whether this finding accounts for the problems in obtaining stable mutants remains to be seen.

Gene transfer in A. vinelandii can take place by transformation (refs. 101, 106, 107) but mapping data has been limited. Page and Sadoff (ref. 101) found that three auxotrophic loci were all linked to a locus conferring rifampicin resistance, and Bishop and Brill (ref. 103) showed that the genes involved in nitrogen fixation (nif) did not fall into one cluster, unlike the nif genes in K. pneumoniae which are closely linked.

In a recent report (ref. 108), RP4, an IncP-1 R plasmid probably identical to R68 (ref. 109) was transferred into A. vinelandii strain UW and was stably maintained. Resistances to ampicillin, tetracycline and kanamycin were all expressed in this background and the plasmid transferred at high frequency between different strains of A. vinelandii. RP4 could also mobilise the multicopy plasmid RSF1010 into A. vinelandii UW and the plasmid could replicate autonomously. This provides the potential for further genetic analysis of A. vinelandii including cloning studies.

Genetic studies on alginate biosynthesis and regulation

Studies on the genetic aspects of alginate biosynthesis in mucoid P. aeruginosa began in 1975, and have been mainly concerned with the regulation of the biosynthetic pathway rather than the pathway itself. No studies have been reported on the genetics of the analogous system in A. vinelandii although non-capsulated mutants have been reported in the context of encystment (ref. 36) and transformation (ref. 101).

Markowitz et al. (ref. 110) attempted to link alginate production with plasmid content in clinical isolates of mucoid P. aeruginosa. They sought evidence of plasmid DNA in cleared lysates from 18 mucoid strains and their isogenic non-mucoid revertants, but were unsuccessful. In addition, the spontaneous loss of the mucoid character was not significantly increased by plasmid curing regimens, e.g. treatment with ethidium bromide, further indicating the lack of plasmid involvement.

Work in our laboratory (refs. 67, 68, 73, 74, 111-114), aimed to test the hypothesis that all wild type P. aeruginosa have the necessary genetic information for alginate synthesis and that this is normally repressed. Hence, mucoid P. aeruginosa would arise following a spontaneous mutation in one of an unknown number of regulator genes. An alternative hypothesis is that wild type

P. aeruginosa have a defective enzyme in the alginate biosynthetic pathway and mucoid strains have a normal one. However, enzyme analysis of a non-mucoid P. aeruginosa (PA0381) described in a previous section (Table 2), suggested that the alginate synthesising enzymes are present, but the specific activities are very low. Thus we have attempted to determine the number and location of genes responsible for the repression of alginate synthesis, prior to identifying the structural genes coding for the relevant enzymes, and determining the nature of the regulatory system(s).

Our approach was based on the elegant studies of Markovitz and his colleagues on mucoid E. coli (reviewed in ref. 115). Wild type E. coli K12 does not appear mucoid when grown at 37°C, however at 30°C, the strain produces an exopolysaccharide composed of glucose, galactose, fucose and glucuronic acid. This polysaccharide is known as colanic acid. Mutant strains of E. coli K12 can be isolated which are mucoid at 37°C, hence the wild type strain contains all the genetic information necessary for colanic acid synthesis, but this is not expressed under these conditions.

Studies on the genetics and regulation of colanic acid synthesis in E. coli K12 began in 1962. This organism was a particularly good candidate for such an analysis because of the wealth of genetic information already available. Early mapping studies revealed that mutations in at least two chromosomal sites yielded mucoid strains. One of these mutations in capR (lon) was shown to be cotransducible with the proC locus using bacteriophage P1, and the second mutation, capS mapped near a trp locus. A third mutation, capT which resulted in colanic acid production on EMB-glucose medium (capR and capS strains are non-mucoid on this medium) at 37°C was also identified. It was possible to confirm the regulatory role of these loci by performing partial diploid studies. Using the F'13 plasmid (a derivative of F carrying the $capR^+$ allele) it was demonstrated that $capR^+$ is dominant over capR and hence $capR^+$ was thought to specify a cytoplasmic repressor protein which acts on the genes for colanic acid synthesis.

A working model for the system, controlling colanic acid synthesis was proposed, i.e. there are several targets on the E. coli chromosome, capR, capS, and capT whose function is to directly or indirectly switch on or off the enzymes involved in colanic acid synthesis. The aim was then to determine whether the control is at the level of transcription or translation and to determine the nature of the regulatory products.

As well as switching on colanic acid synthesis, the capR mutation has a number of pleiotropic effects. CapR strains are UV sensitive, resistant to low levels of tetracycline, chloramphenicol and puromycin and do not allow phages λ and P1 to replicate in the plasmid mode. These properties are not the direct result of colanic acid synthesis, but reflect the diverse role of the

capR gene product. CapS and capT mutants do not share these properties. Recently, (ref. 116) the capR$^+$ gene has been cloned and its product identified as a 94K protein, thought to be under autoregulatory control.

Obviously, our brief outline of the genetic studies on mucoid E. coli has been a greatly simplified version and is not meant to give a detailed account of the regulation of colanic acid synthesis. However, the rationale for these studies in E. coli provided the basis for our approach to the genetics of alginate synthesis in P. aeruginosa, although, at the time of our initial studies, the background genetic information available for P. aeruginosa was far less comprehensive than for E. coli.

The development of the semi-selection technique for isolation of alginate-producing (muc) mutants of P. aeruginosa, already described (Fig. 8), allowed us to put muc mutations into various genetic backgrounds. Table 3 describes the strains relevant to our genetic studies.

TABLE 3

PAO strains used in genetic studies on alginate production

Strain	*Genotype	Origin and/or Reference
PA0381	leu-38, str-2, FP2	ref. 72
PA0568	leu-38, str-2, muc-2, FP2	Group 2 mucoid mutant of PA0381 (ref. 73)
PA0578	leu-38, str-2, muc-22, FP2	Group 2 mucoid mutant of PA0381 (ref. 73)
PA0579	leu-38, str-2, muc-23, FP2	Group 1 mucoid mutant of PA0381 (ref. 73)
PA0585	leu-38, str-2, muc-37, FP2	Group 1 mucoid mutant of PA0381 (our laboratory)
PA0954	met-9011, ami E200, oru-292	Obtained from D. Haas
PA0964	ami-151, hut-C107, pru-354	Obtained from D. Haas
PA01042	pur-67, thr-9001, cys-59, pro-65	ref. 94
PA02021	cys-5605, his-5075, argA171, pro-67, nal-25, muc-36, FP$^-$	Group 2 mucoid mutant of PA02022 (ref. 73)
PA02022	cys-5605, his-5075, argA171, pro-67, nal-25, FP$^-$	ref. 73

*The genotype symbols used are the same as for Fig. 11. The following pairs of markers are closely linked:- ilv-202 and ilvD, leu-38 and leu-8, str-2 and strA, thr-9001 and thr-48, pro-67, pro-65 and proB, nal-25 and nalA. Markers not appearing on the chromosome map are pur, proline utilisation, oru, ornithine utilisation, muc, alginate synthesis.

PA0381 was chosen as the initial genetic background for the mapping of muc loci, as it is a single auxotroph (leu-38), carrying the sex factor FP2 and thus could act as donor in mating experiments.

It should be emphasised at this point that unlike auxotrophic or catabolic markers, muc cannot be selected for in matings or transductions. The small increase in carbenicillin resistance associated with muc is not sufficient to provide a specific selection of recombinants from the large number of recipient bacteria. Hence, the mapping of muc loci is dependent on the demonstration of linkage to known selectable markers.

We postulated that the muc mutation in PA0579 (muc-23) would be located somewhere on the PA0 chromosome, so the strain was used as a donor in plate matings (ref. 117) with various multiply marked recipient strains (non-mucoid, muc^+), selecting for a range of auxotrophic markers in different regions of the chromosome (non-circular at this stage). A significant early result from these matings was that the recombination frequencies for all markers were 10 to 50 fold lower than those obtained using the isogenic non-mucoid donor. However, when a mucoid strain, e.g. PA02021, was used as recipient in a plate mating with PA0381, the numbers of recombinants were not similarly reduced. Later studies involving mucoid donors in plate matings mediated by R68.45 suggested that donorability is not impaired to the same extent using this sex factor. Markowitz et al. (ref. 110) have reported that, in broth matings, alginate synthesis by either the donor or recipient had little or no effect on the transfer of certain R plasmids. The mechanism involved in the inhibition of FP2-mediated chromosome transfer by alginate synthesis has yet to be determined.

When PA0579 was crossed with the muc^+ recipient PA02022, approximately 30% of the recombinants obtained on selection for $cys\text{-}5605^+$ and $his\text{-}5075^+$ were mucoid. These mucoid recombinants were easily distinguished from the non-mucoid variety on the actual mating plates (minimal agar supplemented with the appropriate amino acids), thus showing the same, group 1, characteristics as PA0579. When a similar cross was performed using the group 2 mucoid donor, PA0578, similar linkage of muc-22 to cys-5605 and his-5075 was observed. However, the recombinants had to be transferred to PIA for scoring of muc. This confirmed that the difference between group 1 and group 2 mucoid strains is a result of the mutation leading to alginate production rather than a secondary change modifying the phenotype.

The cross between PA0381 and the mucoid recipient PA02021 (muc-36) likewise indicated linkage of the muc^+ allele to cys-5605 and his-5075. Table 4 shows the results of recombinant analysis.

These results indicated a chromosomal location for muc-36 at a point distal to cys-5605 and his-5075 with respect to the origin of transfer. This was confirmed by interrupted matings (ref. 118) in which cys^+ recombinants obtained at each interruption time were scored for co-inheritance of muc^+, as well as the proximal marker pro^+ and the distal marker arg^+ (Figs. 12a and b).

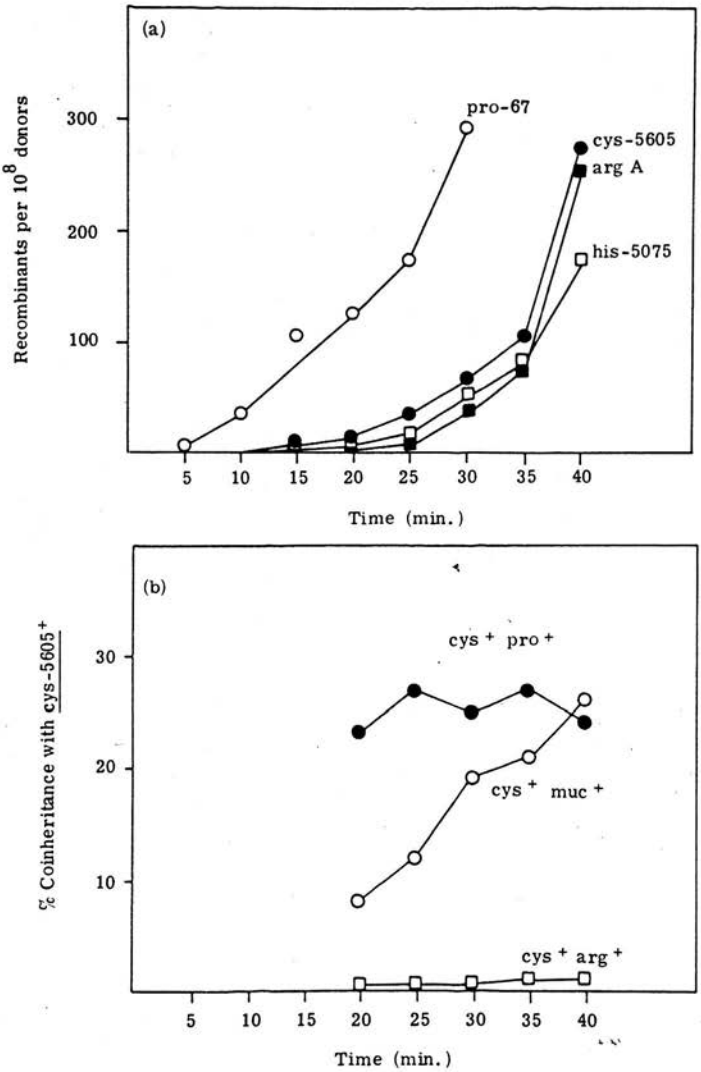

Fig. 12. Interrupted mating between PA0381 and the mucoid recipient PA02021 (muc-36) (a) Time of entry kinetics of pro-67 (○), cys-5605 (●), his-5075 (□), and argA171 (■) (b) Percentage coinheritance with cys-5605+ of the unselected markers muc-36+ (○), pro-67+ (●) and argA171+ (□) (ref. 73).

TABLE 5

Recombinant analysis following plate matings between the donors PA0578 and PA0579 and recipient strain PA01042

Donor	Selected Marker	% Co-inheritance of unselected marker			
		muc	pro$^+$	cys$^+$	pur$^+$
PA0578 (muc-22)	pro-65$^+$	1	-	4	<1
	cys-59$^+$	34	33	-	6
	pur-70$^+$	10	2	5	-
PA0579 (muc-23)	pro-65$^+$	8	-	4	<1
	cys-59$^+$	38	35	-	10
	pur-70$^+$	2	2	4	-

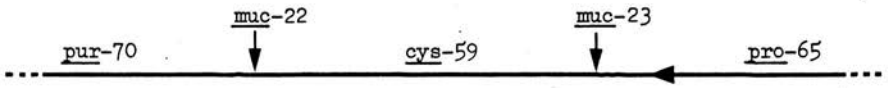

Fig. 13. Approximate locations of muc-22 and muc-23 based on plate mating data.

It has been possible, however, to proceed further with the mapping of muc-22 using transductional analysis, due to the work of Soldati and Haas (manuscript in preparation) who had isolated and mapped several catabolic loci in the region between pur-70 and cys-59 (Fig. 14). Oru-292 and pru-354 are involved in the utilisation of ornithine and proline respectively and show 60% linkage in R68.45-mediated crosses.

Fig. 14. Selectable markers in the region pur-70 to cys-59.

It is extremely difficult to obtain high titres of transducing phage grown in the usual way on mucoid strains, presumably because the phage receptors on the bacterial surface are blocked or the phage immobilised in the viscid environment surrounding the cells. Thus, before muc-22 could be mapped by transduction, it was necessary to isolate a non-mucoid derivative of PA0578 which had arisen due to a second, unlinked mutation switching off alginate production. Such "suppressed" revertants are still able to transfer the

muc allele in matings, giving rise to mucoid recombinants on selection for the appropriate markers. These kinds of strains are relatively common amongst non-mucoid revertants and their nature will be discussed later.

Phage F116L was grown on a revertant of PA0578 and used to transduce PA0954 and PA0964 selecting for oru$^+$ or pru$^+$; transductants were then scored for the co-inheritance of muc-22. 20% cotransduction was obtained between pru-354 and muc-22, while no mucoid transductants were observed on selection for oru$^+$. Similarly, 49% cotransduction was obtained between pru-354 and muc-2 (PA0568).

Four mucoid mutants (all group 2) of PA0964 were isolated and these were transduced with F116L grown on PA01, selecting for pru$^+$. In each case, 30-50% of the transductants were muc$^+$ (i.e. non-mucoid) indicating a similar location for these muc mutations. Two of these strains were used as recipients in three factor crosses to determine the marker order for oru, pru and muc. R68.45 was transferred into PA0954 and this strain was crossed with the two mucoid derivatives of PA0964, selecting for pru$^+$. Recombinants were then scored for co-inheritance of oru and muc$^+$. The results indicated that both muc mutations in these strains were to the right of pru-354 (Fig. 15).

Fig. 15. Marker order determined from 3-factor crosses.

On the basis of results obtained so far, we can conclude that at least two regions on the PA0 chromosome are involved in the repression of alginate synthesis. Mutations in either of these loci (or clusters of loci) result in derepression of the enzymes in the alginate biosynthetic pathway.

The nature of the repression of alginate synthesis in wild type (non-mucoid) strains has still to be determined. Partial diploid studies using R68.45 in a recA background (ref. 119) or involving P. putida (ref. 120) have been reported, and now that at least one muc locus is known to be closely linked to a selectable marker (pru-354), it should be possible to determine whether a cytoplasmic repressor, analogous to the capR gene product, is coded for by this muc locus. Cloning studies in P. aeruginosa have also been reported recently (ref. 121), thus further adding to the techniques available for the genetic analysis of this organism.

Indirect evidence for the protein nature of the capR$^+$ cytoplasmic repressor in E. coli was obtained by growing wild type strains on amino acid analogues, e.g. para-fluorophenylalanine (FPA) (ref. 122). When grown on certain

concentrations of FPA at 37°C, wild type E. coli K12 produced colanic acid, presumably because the analogue was incorporated into the capR$^+$ (or capT$^+$) gene product, resulting in a biologically inactive repressor.

Attempts to find levels of FPA derepressing alginate production in wild type P. aeruginosa have so far proved unsuccessful (ref. 75, Govan and Fyfe unpublished data).

A different approach to the genetic analysis of alginate production in P. aeruginosa has been described by Ohman and Chakrabarty (ref. 123). They attempted to develop a genetic system in a clinical mucoid isolate and map the loci (alg) responsible for the instability of alginate production in this strain.

Using a fluctuation test we have shown that instability in PA0568 can be explained on the basis of spontaneous mutations back to the non-mucoid form (ref. 113). A variety of mutations capable of "switching off" alginate production would be expected, including mutations in the structural genes coding for the biosynthetic enzymes leading to polymannuronic acid, so the approach taken by Ohman and Chakrabarty combined with enzyme analyses would prove valuable in the identification of genes involved in this system.

Ohman and Chakrabarty identified three classes of alg mutants based on colonial appearance and the results of genetic crosses between alg donors and recipients, some of which produced alg$^+$ recombinants. They concluded that the alg loci responsible for each of the three classes of non-mucoid mutants were located in a cluster. Unfortunately, no comparison was made between the markers employed in this system and those appearing on the PAO chromosome map (Fig. 11). Also, no attempt was made to determine the nature of the genes being studied. Hence, it is difficult to relate this work to our studies using PAO.

The possibility that PAO mucoid strains might be genetically distinct from mucoid P. aeruginosa isolated in vivo has been dispelled to a large extent by our finding that a muc locus in the clinical strain 492c (ref. 49) has been mapped in a R68.45-mediated inter-strain cross (with PAO1042) and is located between cys-59 and pur-70 (Fyfe and Govan, unpublished data).

THE SIGNIFICANCE OF BACTERIAL ALGINATE

A taxonomic relationship between Azotobacter and Pseudomonas?

In any consideration of the biosynthesis, regulation and function of bacterial alginate it is difficult to avoid the intriguing and obvious question as to why this unusual polymer, normally associated with marine algae, is uniquely produced in the case of procaryotes by members of two such diverse genera as Azotobacter and Pseudomonas. It is interesting to speculate on the origin, evolution and maintenance of the genes responsible for alginate biosynthesis

and regulation, the biological function of the polymer and even a possible taxonomic relationship between the soil-inhabiting, nitrogen fixing A. vinelandii and the opportunist pathogen P. aeruginosa.

Although comparisons of the genera Pseudomonas and Azotobacter are not commonly found in research publications several studies have recently revealed close relationships between species of these two genera, on the basis of various characteristics. Durham et al. (ref. 124) compared the three enzymes that initiate metabolism of protocatechuate in Azotobacter and Pseudomonas and revealed a close immunological relatedness of isofunctional proteins. Furthermore, they found that in both Azotobacter and Pseudomonas species of the "fluorescent" and "cepacia" groups, β-ketoadipate induces all the enzymes of the protocatechuate pathway (except protocatechuate oxygenase), a regulatory property which sets these organisms apart from other bacteria. The authors concluded that a closely related set of genes may code for the protocatechuate pathway in Azotobacter and Pseudomonas.

While studying the antigenicity of various outer membrane molecules in P. aeruginosa, Hancock et al. (ref. 125) using a monoclonal antibody, demonstrated that a single antigenic site on the major outer membrane lipoprotein, H2, is shared by various strains of P. aeruginosa, P. fluorescens, P. putida and a strain of A. vinelandii. Further evidence for evolutionary conservation of genetic information was provided by De Vos (ref. 126) who has demonstrated significant rRNA homology between A. vinelandii and a variety of fluorescent pseudomonads.

The biological function of bacterial alginate

We have already discussed evidence for a structural function for bacterial alginate in the metabolically dormant microcysts of A. vinelandii. This does not explain, however, the abundant production of extracellular alginate in metabolically active vegetative cells of this species. It is even more difficult to explain the evolutionary retention of alginate biosynthesis and regulation in P. aeruginosa, P. putida, P. fluorescens and P. mendocina. It would seem likely that in vegetative cells alginate has no single function but rather that the polymer, as with other exopolysaccharides (ref. 127), may contribute a range of properties whose specific roles might depend upon the environment. Evidence suggests that, in A. vinelandii, alginate does not serve as an overflow metabolite (ref. 22) but rather that it may act as a protective barrier against heavy metal toxicity (ref. 128), as a diffusion barrier to oxygen (ref. 5), as an ion-exchange system (ref. 129) with enhanced selectivity for Ca^{++}, particularly when alginate is in the gelled state (refs. 38, 130) or provide the bacterium with a hydrophilic, negatively charged coating which provides protection against attack and adverse environmental conditions (refs. 22, 127). Reference has already been made to the possible

utilisation of alginate as a nutrient source in the germination of Azotobacter cysts (ref. 39). Although an alginate lyase can be detected in cultures of A. vinelandii, the biological significance of this enzyme is uncertain. Couperwhite and McCallum (ref. 131) suggested that alginate could serve as a storage polymer although further confirmation for this role has not been found (ref. 22).

In the case of P. aeruginosa, Piggott found no evidence for the production of alginate lyases by mucoid or non-mucoid strains (ref. 75) although alginases have been detected in P. putida and P. maltophilia (ref. 132). No natural, ecological niche for alginate-producing pseudomonads, with the exception of the CF lung, is known. This suggests that in the majority of habitats alginate biosynthesis provides no advantage to the organism. However, in the unusually compromised environment of the CF lung, alginate must confer some advantage. Evidence is now available which suggests a multifactorial role for pseudomonas alginate in CF and provides a classic example of the ability and versatility of P. aeruginosa to act as an opportunist pathogen.

It is interesting to note that although P. cepacia can also be isolated from some CF patients, alginate-producing strains of this species have not been reported. This is in agreement with our observation that alginate-producing P. cepacia could not be isolated in vitro (ref. 70) and suggests that alginate biosynthesis is not a characteristic of this species or, if repressed, is controlled by a mechanism fundamentally different from that found in P. aeruginosa.

The adaptibility of P. aeruginosa and the significance of alginate biosynthesis in this species can be more fully appreciated by a knowledge of the pathological features found in CF patients. CF is an inherited, autosomal recessive disease characterised by symptoms which include high sweat electrolyte levels, pancreatic insufficiency and in the respiratory tract, raised levels of Ca^{++} and Mg^{++} in bronchial secretions which in turn are abnormally viscid due to the presence of highly sulphated glycoproteins (refs. 133, 134). These abnormally viscid bronchial secretions depress the normal non-specific immune defences of the lung in particular the rapid removal of bacteria and foreign matter via the ciliated epithelium of the upper respiratory tract. As we have already discussed in an earlier section of this chapter, during the course of chronic respiratory infection in CF patients, alginate-producing mutants of P. aeruginosa gradually emerge in the majority of patients. The emergence and eventual predominance of alginate-producing pseudomonas correlates with a poor prognosis (ref. 135, 136) suggesting that in CF patients the alginate acts as a virulence determinant. Doggett and Harrison (ref. 51) detected pseudomonas alginate in the sputum of CF patients confirming in vivo synthesis of the polymer. Various studies have suggested mechanisms by which

alginate contributes to the virulence of P. aeruginosa in the respiratory tract, e.g. in bacterial adhesion to the surface of ciliated tracheal epithelium (ref. 137), resistance to attack by host phagocytes (refs. 138-140), resistance to pulmonary surfactant (ref. 67) and to antibiotics (refs. 68, 69) and in reduced pulmonary clearance of P. aeruginosa (ref. 74). To these roles as an adhesin and protective agent it is arguable to add the potential for pathological damage residing in the physical properties of alginate. Aqueous solutions of pseudomonas alginate are extremely viscid and gel rapidly in the presence of physiological levels of Ca^{++} (ref. 74). It is not difficult to imagine the detrimental effect of the polymer's rheological properties in a lung already characterised by sticky bronchial secretions and stasis, and to appreciate, albeit reluctantly, this particular form of bacterial adaptation in a species whose versatility is already well recognised.

FUTURE STUDIES

Many questions remain to be answered concerning the biosynthesis and regulation of alginate in A. vinelandii and P. aeruginosa. Physiological studies have been few and the results obtained dependent to some extent on the strains used. The relationship between cell wall biosynthesis and alginate production has not been fully investigated. In A. vinelandii strain 9068, the rate of alginate synthesis is relatively independent of specific growth rate, suggesting that in these bacteria, competition for precursors between alginate synthesis and cell wall synthesis does not have a great influence on the rate of alginate synthesis (ref. 22). In P. aeruginosa, however, strains which do not produce detectable alginate during log phase, have a higher specific growth rate than those strains exhibiting growth association of alginate synthesis.

No studies have been reported on the genetics of alginate biosynthesis and regulation in A. vinelandii and progress in this area is dependent on further development of the genetic system in this organism.

In P. aeruginosa, the techniques are now available for a detailed analysis of the alginate biosynthetic pathway, along with the added component of its repression in wild type strains. We know that at least two genes involved in the repression of the alginate biosynthetic enzymes are located on the P. aeruginosa chromosome in the region near the major FP2 origin, however, the nature of the gene products has yet to be determined. Partial diploid analysis should reveal whether the muc loci, so far identified, code for repressor proteins or some other regulatory product. Cloning studies will be required if these gene products are to be fully identified.

Yet another question to be answered involves the genetic significance of the different mucoid phenotypes in P. aeruginosa, i.e. groups 1 and 2. In

E. coli, capT mucoid mutants synthesise colanic acid when grown on EMB-glucose agar at 37°C, whereas capR and capS mutants do not. In addition, capR and capS mutants are distinguishable on the basis of other characteristics, e.g. UV sensitivity. Our initial mapping results using PA0578 and PA0579 suggested that strains with mutations at one of the two different sites, (represented by muc-22 and muc-23, Fig. 13) could be distinguished on the basis of alginate synthesis on minimal agar. However, further work has shown that several group 1 mutations are closely linked to pru-354 (unpublished data). The medium component responsible for differential alginate synthesis on minimal agar has not been fully determined. However, we have some evidence that Mg^{++} concentration may play a role, as increased Mg^{++} concentration leads to increased alginate synthesis in some group 2 mutants. No enzyme analyses have been performed on group 2 strains grown in minimal medium, so it is not known at which stage alginate synthesis is blocked.

Apart from increased resistance to various antibiotics and surfactants, indicating cell membrane changes, none of the other pleiotrophic effects detected in capR mutants of E. coli, e.g. UV sensitivity, have been observed in mucoid P. aeruginosa.

Determination of the role of acetylation in the alginate biosynthetic pathway will require the isolation of mutants whose alginate lacks acetyl groups. It will be interesting to see if such alginates have a high guluronic acid content, thus providing evidence for the role of acetylation, postulated by Davidson et al. (ref. 27), to protect certain mannuronic acid residues from epimerisation.

Finally, what of the potential of bacterial alginate as an alternative to the present marine algal product. Clearly bacterial alginates exhibit all the physical properties necessary for the commercial applications of the polymer. The problems may lie in the production of the bacterial polymer on a large scale.

A. vinelandii has been examined in this context and the results reviewed elsewhere (ref. 20). Production of alginate on a large scale by this organism could turn out to be fairly costly as the efficiency of conversion of the carbon source to alginate is low, especially under conditions where respiration rate is high. Genetic manipulation of the organism may overcome this problem.

Amongst the alginate-producing pseudomonads, mucoid P. aeruginosa are strain dependent in respect of the conditions leading to maximal alginate yield. Some strains synthesise alginate during the growth phase, and at first sight would appear good candidates for alginate production in a continuous culture system. However, these strains are extremely unstable and revert back to the non-mucoid state due to the growth advantage of non-mucoid revertants (ref. 113).

Strains which synthesise alginate only in late log phase and stationary phase tend to be more stable, but perhaps more suitable for batch production.

Although advantages in the use of mucoid P. aeruginosa for production of alginate include the relative efficiency of alginate synthesis in terms of carbon conversion, and the possibilities of genetic manipulation, the problems of this organism's potential as an opportunist pathogen must be a major hazard. Alginate producing mutants of P. putida, P. fluorescens and in particular P. mendocina would perhaps be more acceptable, because of these species' relatively infrequent association with infections.

However, physiological studies are required to determine the optimal conditions for alginate synthesis by these species, and any similarity to the genetics and regulation in P. aeruginosa is open to speculation.

It is probable that future studies of bacterial alginate will continue to emphasise the diverse and occasionally conflicting research aims of modern microbiologists. Biochemists and industrial microbiologists will undoubtedly seek to improve the yield, viscosity and gelling potential of alginate as a commercially valuable polymer, especially if supply of the raw source of marine alginate becomes scarce or commercially uneconomic. In contrast, recognising mucoid P. aeruginosa as an important pathogen in the lungs of CF patients, medical microbiologists are already actively seeking means to interfere with alginate production in vivo in order to aid antibiotic therapy and reduce the organism's virulence.

Irrespective of the reasons for studying bacterial alginate, each field of microbiology will certainly benefit from a greater knowledge and understanding of the synthesis, regulation and function of bacterial alginate, both in vitro and in vivo.

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